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A task was assigned to optimize a screening protocol to compare candidate decontamination systems with the dual-component standard system (M258A1) currently fielded to determine the best conditions for experimentation and response expression in decontaminating rabbits exposed percutaneously to sulfur mustard (HD) or Lewisite (L). Five different procedures were evaluated including improved statistics and different methods of lesion evaluation.

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Multiple Animal Studies for Medical Chemical Defense
Program in Soldier/Patient Decontamination and Drug Development

on

TASK 85-01: OPTIMIZATION OF TEST AND RESPONSE CONDITIONS IN A PROTOCOL TO COMPARE THE EFFECTIVENESS OF EXPERIMENTAL DECONTAMINATION SYSTEMS WITH THE M258A1 KIT AGAINST PERCUTANEOUS APPLICATION OF UNDILUTED VESICANT CHEMICAL SURETY MATERIEL TO THE LABORATORY ALBINO RABBIT

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

July, 1988

by

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FINAL REPORT

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TABLE OF CONTENTS

TABLE OF CONTENTS
(Continued)

TABLE OF CONTENTS
(Continued)

TABLE OF CONTENTS
(Continued)

LIST OF TABLES

LIST OF TABLES
(Continued)

LIST OF TABLES
(Continued)

LIST OF TABLES
(Continued)

LIST OF TABLES
(Continued)

LIST OF TABLES
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LIST OF FIGURES

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TASK 85-01: OPTIMIZATION OF TEST AND RESPONSE CONDITIONS IN A PROTOCOL TO COMPARE THE EFFECTIVENESS OF EXPERIMENTAL DECONTAMINATION SYSTEMS WITH THE M258A1 KIT AGAINST PERCUTANEOUS APPLICATION OF UNDILUTED VESICANT CHEMICAL SURETY MATERIEL TO THE LABORATORY ALBINO RABBIT

1.0 INTRODUCTION

A task was assigned to Battelle's Medical Research and Evaluation Facility (MREF) in December 1984 to optimize a screening protocol (MREF Protocol 22) to compare candidate decontamination systems with the dual-component standard system currently fielded by the U.S. Army. A screening regimen was designed to test the variables included in MREF Protocol 22 to determine the best conditions for experimentation and response expression in the elimination of candidate decontamination systems judged not as effective as the M258A1 field kit in decontaminating rabbits exposed percutaneously to sulfur mustard (HD) or Lewisite (L).

A draft protocol (MREF Protocol 23) was submitted in January 1985 to the U.S. Army Medical Research and Development Command's (USAMRDC) Institute of Chemical Defense (USAMRICD) for comment, modification, and subsequent approval for implementation at the MREF. The final protocol, MREF Protocol 23 entitled "Optimization of Test and Response Conditions in the Dermal Study for the Assessment and Validation of Decontaminants in Rabbits Against Mustard and Lewisite," was signed in mid-May 1985, and studies were initiated immediately. A copy of the signed protocol is included as Appendix A.

MREF Protocol 23 contains five areas for evaluation, designated Optimizations A, B, C, D, and E, which are described in Section 2.10 of this report. The experimental designs are based upon MREF Protocol 22, the screening model developed and validated in MREF Task 85-11 (Develop a Screening System(s) for Evaluating Decontaminants of Vesicant CSM, Emphasizing Comparison to the Dual-Component M258A1 System). A copy of MREF Protocol 22 is included as Appendix B.

2.0 EXPERIMENTAL DESIGN

2.1 ANIMALS

Albino rabbits were chosen for this study on the basis of the extensive data base available for percutaneous application of toxic materials in this species and on the size of the application area for multiple challenges with neat chemical surety materiel (CSM). Equal numbers of 2.0- to 4.0-kg male and female New Zealand White (albino) rabbits from the Kings Wheel Rabbitry, 8085 Camp Road, Rt. 5, Mt. Vernon, Ohio 43050, were assigned to treatment groups based on body weights. Preselections were made on all rabbits to obtain only those with hair growth patterns that would allow bilateral, pair-wise comparisons of standard and candidate dosing sites. All animals were quarantined for at least 7 days at Battelle's Animal Resources Facilities at 505 King Avenue before being transported to the MREF.

Upon receipt at the Animal Resources Facilities, the rabbits were ear tattooed for positive identification, weighed, sexed, and observed for signs or symptoms of disease. At the MREF, animals were acclimated for at least 24 hr prior to being placed on study. At both facilities, housing was individual in stainless steel, slotted cages equipped with automatic watering systems. Humidity was programmed at 50 percent (±10 percent) and temperature at 70 F (±5 F). Fluorescent lighting was maintained at a light/dark cycle of 12 hr each per day. Purina Certified Rabbit Chow and water were available at all times during quarantine and holding. During the 24-hr test period, animals were given free access to water but were not given rabbit chow while in treatment stanchions.

Battelle's physical facilities are of appropriate size, construction, and location for the conduct of nonclinical laboratory studies. The animal resources facilities have been registered with the U.S. Department of Agriculture as Research Facility Number 31-R-21 since August 14, 1967, and are periodically inspected in accordance with provisions of the Federal Animal Welfare Act (Public Law 91-579 and subsequent revisions) and the "Guide for the Care and Use of Laboratory Animals," NIH Publication No 85-23, Revised 1985.

A statement of assurance regarding the Department of Health, Education, and Welfare policy on humane care and use of laboratory animals has been filed with the Office for Protection from Research Risks, National Institutes of Health. The letter of acceptance is dated July 29, 1986 for assurance number A3034-01. In addition, the Council on Accreditation of the American Association for Accreditation of Laboratory Animal Care (AAALAC) granted the Battelle animal care program full accreditation on January 31, 1978. This was renewed on July 11, 1980, July 1983, and December 1986.

2.2 TREATMENT DESIGN

The optimization steps described later in Section 2.10 make use of the basic model developed in MREF Protocol 22. Specific deviations from the basic protocol are described for each optimization step in the appropriate section. The basic model is described below.

Three groups of eight rabbits (four male and four female) were matched by weight after selecting animals with suitable hair growth patterns within the dorsal application area. Each animal in the group received a series of 0.5-µl applications of CSM along the dorsum of the back in the following pattern:

Anterior ← → Posterior

Midline	S1	S2	S3	S4
	N1	N2	N3	X

N = 0.5 µl of CSM followed by experimental candidate decontamination system

S = 0.5 µl of CSM followed by M258A1 I and II (in sequence) standard decontamination system (except at S4)

X = No treatment or challenge

1 = Decontamination at shortest time period

2 = Decontamination at middle time period

3 = Decontamination at longest time period

4 = CSM without decontamination.

Each dose/decontamination area was approximately 100 mm long (laterally from the midline) and 25 mm wide.

2.3 EXPERIMENTAL COMPOUNDS

The materials used to test each optimization step were both components of the M258A1 field kit. The dual-component M258A1 kit was chosen, in consultation with USAMRICD representatives, for use as the standard decontamination system. In some cases, the effectiveness of the system was ascertained using distilled water, which was selected as a liquid material that would not be effective as a decontaminant against percutaneous application of HD or L.

The M258A1 kit consists of two components (I and II) to be used in sequence in field application as specified by the kit use instructions (TM 3-4230-216-10, April 1982). Each component is individually packaged in aluminum foil to maintain activity of the ingredients and to prolong the storage life of the kit.

Component I consists of a nominal 2.75-inch by 5-inch towelette moistened with 3.5 to 4.9 g of decontaminating solution (average volume estimated to be 4.25 ml). The decontaminating solution is a mixture of ethanol (72 percent w/w), phenol (10 percent w/w), sodium hydroxide (5 percent w/w), ammonia (0.2 percent w/w), and water (12.8 percent w/w) in accordance with Military Specification DOD-D-51467(EA), 25 February 1980 and Military Specification MIL-D-51468(EA), 11 August 1983. Component II consists of a nominal 2.75-inch by 5-inch dry towelette impregnated with 1.0 g of chloramine B (quantity to produce an active chlorine content of 0.156 g) and three crushable glass vials containing a total of approximately 4.5 ml of a mixture of ethanol (45 percent w/w), zinc chloride (5 percent w/w), and water (50 percent w/w) in accordance with Military Specification DOD-D-51467(EA), 25 February 1980; Military Specification DOD-C-51464(EA), 25 February 1980, and Military Specification MIL-D-51468(EA), 11 August 1983.

Individually wrapped and prepared kit components were not used in the validated screen in MREF Protocol 22. Instead, the kit components were made of towelettes from bulk rolls and solutions from bulk containers fresh at the moment of use in the experiment. The bulk liquid solutions were applied

directly to the precut towelettes in the hood immediately prior to in-hood use to minimize personnel exposure and evaporation of the volatile portions of the liquid solutions. The volumes of the liquid portions and sizes of the cloth towelettes used in these experiments were proportional to the specifications that governed their manufacture (Military Specification DOD-C-51464(EA), 25 February 1980; Military Specification DOD-D-51467(EA), 25 February 1980, and Military Specification MIL-D-51468(EA), 11 August 1983). The components of the M258A1 system were obtained from Chemtronics Corp., Swannanona, North Carolina, and were used in screens performed before mid-April 1986. After that, the M258A1 components were obtained from Mine Safety Appliances, Murraysville, Pennsylvania.

The L and HD were supplied by USAMRICD, and the following information was obtained from USAMRICD for each:

	HD	L
Lot No.	39131-4	39135-4
Purity (%)*	97.3	95.8
Density (g/ml)	1.27	1.88
Known impurities		
Cis isomer		4.0
Diathane	1.2	
Unknown Impurities	1.5	0.2
Additives	None	None
Color	Colorless	Colorless
Appearance	Clear liquid	Slightly oily liquid.

*All percentages are calculated by weight and are subject to ±0.2 variation due to analytical imprecision.

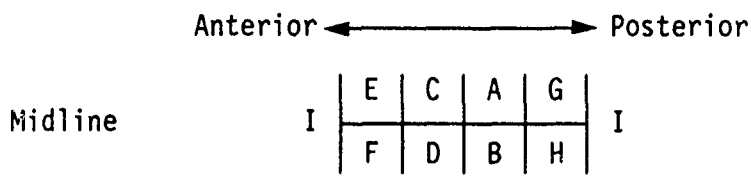
Battelle did not confirm the purity, density, impurities, or additives information supplied by USAMRICD. Dose analyses were not performed since CSM was applied undiluted.

2.4 PREPARATION OF ANIMALS

Prior to application of CSM, each rabbit was clipped and anesthetized with a 3.5:1.0 (w/w) mixture of ketamine and xylazine (17.5 mg/kg and 5.0 mg/kg, respectively) by intramuscular injection. Dosing areas were marked on the dorsum with a felt-tipped pen. The unconscious animals were then placed in stainless steel stanchions and transported to the hood for dosing.

2.5 APPLICATION OF VESICANTS

A constant dose of 0.5 μl of CSM was chosen based on the validation results of MREF Protocol 22. The CSM was applied to each of the seven sites on the back of each rabbit (see Section 2.2, Treatment Design) as a small streak approximately 100 mm in length and 20 mm in width perpendicular to the spine with a Hamilton 7001 1.0-μl syringe. L was applied with a special Hamilton 7001 syringe equipped with a platinum barrel and a tungsten plunger, while HD was applied with a standard stainless steel syringe. The following pattern of application was used on each rabbit:



- Application of CSM proceeds from A-G in alphabetical order to allow proper sequencing of timed decontaminations at each site.
- Applications at site H and at site I are done during the dosing regimen at the first available time period.

2.6 DECONTAMINATION

The M258A1 decontamination components were applied in field-use sequence to the vesicant-dosed areas as described for each optimization step. Application of component I for 5 sec began at 1.25, 5.0, or 10.0 min after administration of HD dose or at 30, 60, or 120 sec after an L dose.

Application of component II for 10 sec began 65 sec later. The application regimens were those validated for MREF Protocol 22, which were based on the field-use instructions for the M258A1 kit.

Applicators for component I and component II were made by cutting bulk M258A1 I or II kit cloths to an area of approximately one-half the kit pad size, taping it around one end of a tongue depressor, and wetting it immediately prior to use with 2.25 ml of either I or II bulk decontamination solution. The component I applicator was wiped over the dosed area briskly but not harshly at approximately three strokes per second for a total of 5 sec. The strokes were made within the approximate 100 x 25 mm dose/decontamination area with a lateral motion perpendicular to the spine. Only one side of the applicator was wetted and used. The component II applicator was applied to the dose/decontamination area in a fashion similar to component I, but for a total of 10 sec. Both sides of the component II applicator were wetted (2.25 ml total) and used (10 sec total), which made full use of the chloramine B powder impregnated in the component II cloth.

2.7 LESION EVALUATION

Immediately prior to lesion evaluations at 20 to 24 hr after dosing, the dose sites were washed off with 5 percent sodium hypochlorite (NaClO; bulk commercial grade diluted with distilled water) solution followed by three distilled water rinses.

Three criteria of lesion size (length, width, and area) were measured to determine the effectiveness of the candidate decontamination system as compared to the M258A1 standard system. Visual estimates of lesion length and width were made by reference to a plastic metric ruler. Lesion length was defined as the longest dimension of the lesion (in millimeters), generally in the shape of an ellipse. Width was defined as the greatest distance (in millimeters) from one side of the lesion to the other side along an axis perpendicular to the length. Area (in square millimeters) was calculated as 0.25π times the product of length and width, assuming an ellipsoid. Dimensions of lesion shapes that clearly indicated a noncontinuous dose application were recorded but were not included in the data analysis.

The ease of estimation of lesion length and width was enhanced by intramuscular injection of 1 ml of a 3 percent suspension of trypan blue dye in saline into each thigh of the rabbit approximately 2 to 4 hr before lesion length evaluations. Animals were anesthetized with a mixture of ketamine (17.5 mg/kg) and xylazine (2.5 mg/kg) just prior to lesion evaluations. Photographs were taken of the lesions to supplement the estimates, if necessary.

After lesion evaluation at 24 to 28 hr after exposure, the rabbits were killed by administering T-61, a euthanasia solution.

2.8 NECROPSY AND HISTOPATHOLOGY

No tissue samples were saved and all animal carcasses were decontaminated and discarded.

2.9 STATISTICAL ANALYSIS

The lesion size data analyses included graphical displays, summary statistics, and application of analysis of variance (ANOVA) methodology to estimate biologically important contrasts among the measured lesion sizes and to determine their statistical significance. Scatterplots and summary statistics of average lesion lengths, widths, and areas provided direct visual comparisons of the effects observed from each test. The ANOVA methodology used a multifactor model that reflected two sources of experimental variation: animal-to-animal variation and within-animal variation. Since different animals were placed on study in each of the three replicate days, comparisons among days were based on comparisons across animals; thus, they incorporated animal-to-animal variation as well as within-animal variation.

2.10 OPTIMIZATION STUDIES

This report presents the data obtained and analyzed for the five optimizations described in detail below. Analyses of data collected from each optimization are presented and discussed as to the significance of the finding relative to the standard protocol step being evaluated.

2.10.1 Optimization A: Removal of Animals from Hoods on Day of Dosing

Much of the time and effort in the MREF Protocol 22 decontamination system screen is due to the retention of rabbits in the dosing hood for periods of 24 to 28 hr after dosing. The conservative safety assumption has been that evaporated vesicant exists at hazardous levels in the air surrounding the dosed rabbits during this period. This assumption increases the time and effort of each screening study by making the hoods unavailable for any other dosing work, requiring extensive cleanup of animal wastes that have accumulated in the hoods overnight, requiring proof of decontamination of animal carcasses, and making evaluations of lesions at 24 to 28 hr after dosing cumbersome and difficult because they must be conducted within the hood system.

The current requirement to maintain the dosed animals in the hood system for the duration of the study necessitates that the hood system be dedicated to each screening run for 2 consecutive days, one day for dosing and one for assessment of lesions and extensive decontamination of animals and hoods. Therefore, only two screening runs can be performed in a hood system in a normal work week.

The ability to remove the dosed animals from the hoods on the same day as dosing would make the hood system available for next-day use, decrease the effort needed for hood cleanup, render proof of animal decontamination unnecessary, and allow evaluations of lesions to be made outside of the hood system in an examination room.

These incentives prompted us to examine the consequences of an early decontamination and removal of animals from the hoods:

- The potential hazards to personnel from vesicant evaporating from animal backs after earlier decontamination with NaClO solution
- The effect of decontamination with the NaClO solution and rinsing with distilled water at 4 hr after dose on local irritation and lesion size at the 24- to 28-hr time for lesion size estimations.

Four hours was selected as the exposure period because it was consistent with U.S. Army Chemical School Nuclear, Biological, and Chemical (NBC) hazard protection and decontamination doctrine as a typical duration that a vesicant-exposed soldier would likely experience in the battlefield before rendezvous with chemical decontamination squads (Final Draft, FM 3-5, NBC Decontamination, August 31, 1984, U.S. Army Chemical School).

2.10.1.1 Optimization A: Experimental Design

Rabbits were tested in subgroups of eight per day. These test animals received 0.5 µl of HD or L at seven dose sites and were decontaminated at three different times after dosing. Right-side dose sites were decontaminated with M258A1 components I and II per MREF Protocol 22, and left-side dose sites were decontaminated twice with distilled water at the time sequences used for M258A1 components. This scheme was used to simulate conditions for maximal presence of vesicant when performing the decontaminant system screen, as would be the case when a candidate system no more effective than water was tested against M258A1 I and II. All seven sites were decontaminated at 4 hr after dosing with a NaClO solution, followed by three rinses with distilled water. The concentration of the NaClO solution was varied to determine the highest concentration that would not produce an increase in the generalized erythema of the decontamination sites at lesion readings 24 hr after dosing.

A series of prevalidation studies were conducted with HD to determine the highest reliable nonirritative concentration of NaClO. These studies showed that 0.5 percent concentration of NaClO did not increase the erythema or edema caused by HD exposure. In addition, several studies were conducted using either one or two animals to determine the presence of HD sampled after no decontamination and to verify the ability of the sampling method to collect HD vapors.

Three runs were then conducted with HD (24 animals total) and two runs with L (16 animals total) to assess the safety and lesion size effects of the early decontamination with 0.5 percent NaClO. After NaClO decontamination and three distilled water rinses, the rabbits were individually placed in stainless steel stanchions within cardboard boxes (with the tops removed) in

the dosing hood. The boxes were used to reduce the air velocity in the vicinity of the rabbit back, thereby minimizing vesicant vaporization.

A large plastic funnel was cut to the curvature of the rabbit's back and positioned over the dosed sites. Tygon tubing was attached to the small end of the funnel. This assembly, supported by a ring stand with clamp, was developed to replace the plastic bags described in MREF Protocol 23 (Appendix B, page 9). The change to funnels was made to decrease the possibility of false negative results from decontamination by water evaporated from the rabbit back. Perspiration by the rabbits was noticeably reduced by using the funnel assembly.

Headspace air over the dosed sites was sampled at a rate of 2.0 liters/minute (l/min) through impingers filled with 10 ml of ethylene glycol diacetate (EGDA). Sample periods were 3 hr for HD and 2 hr for L. The sample solution was analyzed either by gas chromatography (GC) (for HD) or by atomic absorption spectroscopy (for L). Results were compared with the 10-day time-weighted average (TWA) established for each vesicant by USAMRICD for Battelle under Contract No. DAMD17-83-C-3129.

Estimates of lesion length and width were made at 24 to 28 hr after dosing. Lesion length was the primary response. Contralateral comparisons were made at each time to decontamination as in the base protocol. This experimental model was similar to the MREF Protocol 22 validation studies reported in MREF Task 85-11, 20 November 1985. The only difference was that this study used a 4-hr decontamination with 0.5 percent NaClO instead of a 24-hr decontamination with 5 percent NaClO. Thus, we compared the results from this model with results obtained from the initial validation of MREF Protocol 22 for HD.

Under MREF Task 85-11, an attempt to validate MREF Protocol 22 had failed at times to decontamination of 1.25, 5.0, and 10.0 min. The contralateral difference in lesion lengths resulting from HD decontaminated with distilled water versus M258A1 I and II at 10 min after dosing was not significant ($P > 0.05$, one-sided). Times to decontamination were reestablished at 1.0, 3.0, and 5.0 min and validated under MREF Protocol 22. This validation occurred after Optimization A had been performed with HD. A re-test at these new times in MREF Protocol 23 to establish the safety of the 0.5 percent NaClO

decontamination and removal of animals from the hood at 4 hr after dosing was deemed unnecessary since measurements taken at 1.25 and 5.0 min in the Optimization A series showed no measurable HD concentration present.

For L, times to decontamination with either M258A1 I and II or distilled water were 30, 60, and 120 sec. The model was tested for safety and for the lesion size effect of decontamination with 0.5 percent NaClO at 4 hr after dosing versus 5 percent NaClO at 24 hr. The results of this test with 16 animals were compared to the MREF Protocol 22 validation of L decontaminated with M258A1 I and II as reported in MREF Task 85-11.

2.10.1.2 Optimization A: Statistical Analysis

During the validation of MREF Protocol 22, contralateral contrasts (one-sided, paired t tests at alpha = 0.05) were performed between lesion size estimates obtained by decontamination with either M258A1 I and II or distilled water followed by decontamination at 24 hr with 5 percent NaClO. Significance of the contrasts was based on an ANOVA model, incorporating both between-animal and within-animal variation. Under Optimization A, contralateral contrasts were similarly performed between lesion size estimates obtained by decontamination with either M258A1 I and II or distilled water followed by decontamination at 4 hr with 0.5 percent NaClO. For each NaClO decontamination scheme, these contralateral contrasts (i.e., M258A1 I and II versus distilled water) were performed at each time to decontamination and on all paired data pooled across times to decontamination. In order for the early NaClO decontamination to be an acceptable modification, the contralateral difference in lesion length between M258A1 I and II and distilled water had to be significant at each time to decontamination. This criterion for accepting the modification was adopted in consultation with USAMRICD, and ensured that the procedure retained the ability to discriminate between good and poor decontamination systems after modification.

We also wanted to know whether the screen had been significantly changed by the early NaClO decontamination procedure. To test this, the contralateral differences were contrasted (late less early NaClO decontamination, two-sided, unpaired t test at alpha = 0.05) at each time to decontamination

and for the data pooled across times to decontamination. The t score, i.e., the studentized difference between the contralateral differences for the two NaClO decontamination schemes, was interpreted as an index of the degree of change in the screen model. A positive t value with $P < 0.05$ indicated that the early NaClO decontamination scheme had increased the difference between lesion sizes for the standard versus a nominally inadequate decontamination system. That is, a significant positive t value meant that the sensitivity of the model had been improved by the early decontamination.

The procedure was performed for the HD screen at 1.25, 5.0, and 10.0 min to decontamination, and for the L screen at 30, 60, and 120 sec to decontamination.

2.10.2 Optimization B: Positional Effects in Test Design

Studies performed in January 1984 established an experimental design under Task 84-1 (Vesicant Screening Protocols for Decontaminants) for the general decontamination screening system. Those studies did not determine the effects of vesicant dose position on the rabbit's back on lesion size and of how much the decontamination process varied with dose position. It was suspected that an anterior-posterior gradient in the thickness of the epidermis and/or dermis of the albino laboratory rabbit might be enough to affect the lesion size response. Optimization B was designed to test for the histologic variables implicit in the screen design, as well as variables inherent in the decontamination process itself (i.e., how each dosed area is presented to a nonambidextrous decontamination technician and how accessible that area is with respect to side and the distance the technician must reach inside the hood to apply the decontamination system). Optimization B evaluated the total expression of these variables.

2.10.2.1 Optimization B: Experimental Design

The effect of the position of the dose was examined using a three - way factorial experimental design. The factors concurrently evaluated were side (left/right), anterior-posterior position (from 1 = most anterior to 4 =

most posterior within the nominal treatment region of the shaved dorsum), and time to decontamination (at 1.25, 5.0, and 10.0 min and 24 hr, and in a later study at 1.0, 3.0, and 5.0 min and 24 hr for HD; and at 30, 60, and 120 sec and 24 hr for L). A set of 8 animals with the same ordering of time to decontamination with anterior-posterior position was designated a group. The factors position, group, and time to decontamination formed a replicated 4 x 4 Latin square, with eight replications. These factors were completely crossed with side, a two-level factor. Animal was used as a blocking variable, with 4 of the 16 combinations of position and time to decontamination run on each animal. Each animal was dosed and decontaminated in the same manner with M258A1 I and II on both sides at each position while we varied the time to decontamination from group to group. Thirty-two animals in four groups of eight animals each were used per study. The animals for each group were divided over 2 days of testing per study, i.e., 16 animals per day. Group definitions were as follows:

			Position			
Group	N	Side	1	2	3	4
1	8	Both	S	M	L	ND
2	8	Both	M	L	ND	S
3	8	Both	L	ND	S	M
4	8	Both	ND	S	M	L

where

S = shortest times to decontamination

M = middle times to decontamination

L = longest times to decontamination

ND = no decontamination until just before lesion assessment at 24 hr after dosing, then with 5 percent NaClO solution followed by three distilled water rinses.

All other experimental details of dosing, decontamination, and lesion size estimation were the same as in MREF Protocol 22. Lesion responses were length, width, and calculated area, assuming an ellipsoid lesion shape.

2.10.2.2 Optimization B: Statistical Analysis

Lesion estimates were averaged (n = 8) by group across both days per study. Each mean represented the lesion response (length, width, or area) for a vesicant dose applied at one of four anterior-posterior positions, one of two sides, and at one of four times to decontamination. The estimates were submitted to a three-way ANOVA that evaluated the primary effects of position, side, and time and all possible interactions of these. Calculations for Type III sums of squares, which do not assign priorities to factors, were performed. Estimates of contralateral differences at each position (pooled across times) and separately at each time (pooled across positions) were calculated along with t statistics and probabilities for the null hypotheses. These estimates were calculated since side was the factor of primary concern. Tests for linear and quadratic trends of lesion size with position or time were also conducted. Results of the statistical tests were interpreted as indices of the validity of the model for comparing the standard versus candidate decontamination systems against vesicants at different exposure periods.

2.10.3 Optimization C: M258A1 Standard Kit Materials Packaging

Both MREF Protocol 22 and its precursor, MREF Protocol 1 (screen for decontaminants using single, most effective M258A1 component), used standard materials from bulk packaging to make the M258A1 decontaminating systems instead of using the actual foil packets from the field kit. Preparation of standard materials from bulk was preferred by USAMRDC and Battelle for economy, ease of preparation, and safety to operating personnel.

2.10.3.1 Optimization C: Experimental Design

Optimization C was designed to determine whether there was any difference in lesion response to M258A1 decontaminant obtained from bulk versus kit packaging. The experimental model was the same as for MREF Protocol 22, using both components of the M258A1 standard. Twenty rabbits each were used

for HD and L. Right-side dose sites were decontaminated with M258A1 materials prepared as in Section 2.6. Left-side dose sites were decontaminated with M258A1 kit (manufacturer's date: February 1985, Lot 001) materials taken from freshly opened packets and taped around wooden tongue depressors. Kit instructions were followed for preparing component II, which called for crushing glass vials to prewet the application pad before opening the packet. All other experimental details were identical to MREF Protocol 22. MREF Protocol 23, page 11, directs the test to be performed at only the shortest time to decontamination (1 min for HD; 30 sec for L). After consultation with USAMRICD, a decision was made to use the entire animals' backs and to compare M258A1 bulk versus kit packaging at 1.0, 3.0, and 5.0 min for HD and at 30, 60, and 120 sec for L. The most posterior site on each side (G and H) was dosed and decontaminated 24 hr later with a 5 percent NaClO solution followed by three distilled water rinses. The G and H sites served as controls for a possible position (side) effect, since the results of Optimization B were not known at the time.

2.10.3.2 Optimization C: Statistical Analysis

Statistical methods included one-sided, paired contralateral contrasts to test the null hypothesis that there was no difference between the use of bulk and field kit foil packets. This one-sided experimental design was used to simulate a MREF Protocol 22 screen in which the M258A1 bulk components served as the usual standard decontamination system and the M258A1 field kit foil packet components served as the system being tested.

2.10.4 Optimization D: Optimization of Times to Standard Decontamination

Two of the three variables associated with the M258A1 kit, amount of decontaminant and duration of wiping, are fixed by the M258A1 standard kit and the instructions for its use (TM 3-4230-216-10, April 1982). The remaining factor, time to decontamination, was varied in Optimization D to determine three exposure durations that would result in lesion sizes of approximately

25, 50, and 75 percent of an unchallenged control dose applied contralaterally to each. Since times to decontamination for L were already short due to its nearly instantaneous necrotizing effect, this optimization was performed for HD only.

2.10.4.1 Optimization D: Experimental Design

Six dose sites were used on the backs of eight animals per run. Exposure periods were varied on the three left-side dose sites from run to run. Left-side HD dose sites were decontaminated with M258A1 I and II per MREF Protocol 22, and right-side dose sites were not decontaminated. All lesion sites were washed with 5 percent NaClO solution followed by three distilled water rinses at 24 hr after dosing. Relative lesion size (R_L) was expressed as a ratio of the left-side lesion length (L_t) to its contralateral non-decontaminated right-side lesion length ($L_{24\ hr}$). A scattergram of relative lesion length versus time to decontamination was plotted.

Times to decontamination in these studies ranged from 10 to 75 sec, which were generally shorter than the times that had been validated under MREF Protocol 22 for screening candidate decontamination systems. In order for a lesion growth curve generated by these data to contain information beyond 75 sec to decontamination, we decided to include MREF Protocol 22 validation data. Those validation studies had been conducted at two sets of three times to decontamination, i.e., at 1.25, 5.0, and 10.0 min and at 1.0, 3.0, and 5.0 min, which offered six more sets of points for the lesion growth curve. The data were corrected for anterior-posterior positional effects evident from Optimization B before being included in the lesion growth scattergram.

2.10.4.2 Optimization D: Statistical Analysis

The relative lesion length data obtained under Optimization D of MREF Protocol 23 and the data obtained in the validation of MREF Protocol 22 were separately subjected to outlier tests at each time period. The two-sided method of Grubbs (1969)[3] was used at alpha = 0.05. The method was incorporated into a SAS (Statistical Analysis System, Inc., Cary, NC) algorithm that

input the data at each time period as a univariate sample and calculated studentized residuals in a single-parameter regression model. The program then identified and eliminated the most extreme outlier (if any) in either tail. The procedure repeated itself until no outliers remained.

Since the outlier test was sensitive to non-normality in the data distributions, the arcsine ($x^{1/2}$) transform, which is commonly used to normalize fractional parameters, was employed, i.e.,

$$y = \text{arcsine}\left(\sqrt{\frac{R_L}{M + 0.01}}\right) ,$$

where

y = variable subjected to the outlier test,

R_L = left-side lesion length/24-hr control-side lesion length, and

M = either the maximum R at the given time to decontamination or 1.00, whichever was greater.

Division of R by either the sample maximum plus 0.01 or 1 plus 0.01 was necessary to assure a valid argument for the arcsine operation.

With outliers deleted, the data from MREF Protocol 23 Optimization D and from MREF Protocol 22 HD validation studies were combined for calculations of the regression parameters in the negative exponential growth function

$$R_L = 1 - B_2 e^{(-B_1 t)} ,$$

where

R = ratio of the lesion length to the 24-hr control value,

e = Naperian base, 2.718,

B_1 = exponential regression parameter,

B_2 = fractional complement to the y-axis intercept (B_2 = 1-intercept), and

t = time in seconds after dosing.

An iterative regression analysis was performed using a SAS-weighted nonlinear regression procedure (NLIN) with the Marquardt (1963)[6] least-squares method. The rationale for employing the above model was the inverted decay-type shape of the points in the scattergram of the combined data. The

parameters B_1 and B_2 were allowed to float in iterative, weighted least-squares calculations that moved toward a minimal sum of squares. The weighting factor was $1/(R)^2$. The only restriction in the model was that, at infinite time after dosing, the relative lesion length was forced to 1.0 (the first term in the right side of the equation). This was approximately equivalent to defining that each lesion, if not decontaminated, would equal the 24-hr control in lesion length at t = 24 hr.

As shown in Section 3.4.3, relative lesion length was shown to be an unsatisfactory response because it did not regress to a level less than 0.25. The intent of the optimization had been to define new times to decontamination as exposure periods at which relative lesion length approximated 25, 50, and 75 percent by interpolation of the curve. We decided to investigate another parameter, relative lesion growth (R_G), defined as

$$R_G = \frac{L_t - 10\ \text{mm}}{L_{24\ \text{hr}} - 10\ \text{mm}} \quad ,$$

where

L_t = lesion length at time t after dosing and

$L_{24\ \text{hr}}$ = lesion length at 24 hr after dosing.

The target application length, 10 mm, was subtracted from both numerator and denominator. The data for this response contained two nonpositive members so that the square root operation could not be performed in the arcsine ($x^{1/2}$) transform preparatory to the outlier test. Thus, results from the outlier test performed on R_L were used to identify outliers. Negative members were treated as zeros in the regression analysis. The same least-squares regression model was used as before. The weighting factor was changed to $1/(R + 1)^2$ to prevent extreme weighting of members equal to or approximating zero. Times to decontamination were determined at R = 0.25, 0.50, and 0.75 by solving for t in the negative exponential equation, i.e.,

$$t = \frac{\ln\left(\frac{1-R_G}{B_2}\right)}{-B_1} \quad .$$

2.10.5 Optimization E: Improved Skin Irritation Evaluation

The screen developed in MREF Protocol 1 used classical Draize (1944)[2] irritation scoring to test the ability of candidate decontaminants to reduce erythema and edema. Work performed under Task 84-7 showed this method to be unsuited for evaluating severe dermal irritation, such as that due to vesicant CSM exposure. The Draize method was not able to distinguish degrees of irritation among the standard and six candidate decontaminants. That is, group mean erythema and edema were severe in all groups at the dose levels and exposure times chosen for the screen.

Optimization E involved the investigation of several optical and computer technologies as possible means of devising a workable procedure for scoring severe skin irritation. This was done so that the logic of making procedural changes based on previous results would be clearer.

3.0 RESULTS AND DISCUSSION

Tables are presented in Appendix C and Figures are presented in Appendix D.

3.1 OPTIMIZATION A

Results of Optimization A studies for HD and L are presented in the following sections. Data from the volatilization studies are presented in Table 3.1.1. Tables 3.1.2 through 3.1.10 and Figures 3.1.1 through 3.1.6 present data for 4- and 24-hr NaClO decontamination comparisons of HD. Tables 3.1.11 through 3.1.19 and Figures 3.1.7 through 3.1.12 present similar data for L.

3.1.1 Determination of Volatilized HD

Preliminary work in the MREF showed the highest effective but consistently nonirritative NaClO solution concentration was 0.5 percent. The detection limit of HD by GC in the MREF is 0.6 ng/l, or 20 percent of the TWA (3.0 ng/l) established for HD at Battelle under Contract No. DAMD17-83-C-3129. Analysis of the EGDA samples collected through funnels placed over the dosed sites revealed no detectable HD from any of the 24 animals used in the three studies. Results were reported as less than 0.2 TWA.

Table 3.1.1 includes results from two single-animal studies that demonstrated no detectable HD at 4 hr after dose even without NaClO decontamination. HD was not detected either when the 0.5 percent NaClO solution step was omitted or when all steps of mechanical removal were omitted. It was apparent that 4 hr of evaporation from and penetration through skin was enough for the dissipation of 0.5 μl of HD applied percutaneously.

The ability of the method to collect HD was ascertained by initiating the sampling period at 7 or 11 min after dosing of 0.5 μl of HD at each of seven sites. Analyses from two rabbits sampled at these time periods showed HD concentrations of 2,740 and 880 ng/l of air, which are equivalent to recoveries of 22.2 and 7.1 percent of the total dose as calculated below:

$$\% \text{ Recovery} = \frac{[HD] \cdot T \cdot R \cdot 100\%}{n \cdot v \cdot d \cdot c} ,$$

where

[HD] = average concentration of HD in nanograms per liter of air sampled,

T = sampling period (180 min),

R = sampling flow rate (2.0 l/min),

n = number of dose sites (seven per animal),

v = dose volume per site (0.5 μl),

d = density of HD (1.27 mg/μl), and

c = conversion factor (10^6 ng/mg).

These tests served as positive controls for the funnel collection method.

The results of these studies indicated that decontamination of percutaneously applied HD to the backs of rabbits with 0.5 percent NaClO (followed by three distilled water rinses) at 4 hr after dosing eliminated offgassing of HD to nondetectable levels by GC analysis. Therefore, the removal of animals from the hoods after this decontamination process should not present undue hazards to laboratory personnel, since the offgassing of HD from the backs of animals is below detectable limits and substantially below the permissible HD exposure limits.

3.1.2 Determination of Volatilized L

The detection limit of arsenic in the MREF by flameless atomic absorption spectroscopy (FAAS) is 1.15 ng/l, or 38 percent of the TWA (3.00 ng/l) established for L. Results of two studies using eight rabbits per study are presented in Table 3.1.1. Analysis of the EGDA samples collected through funnels placed over the dosed sites revealed arsenic concentrations below the TWA of L for all 16 animals in both studies. Results from the second study showed all arsenic levels were also less than the detection limit.

These data demonstrate that decontamination of percutaneously applied L to the backs of rabbits with 0.5 percent NaClO solution (followed by two distilled water rinses) at 4 hr after dosing eliminated offgassing of L and its arsenical derivatives to safe occupational exposure levels. Therefore, the removal of animals from the hoods after this decontamination process should not present undue hazards to laboratory personnel, since the arsenic levels are below the permissible L exposure (TWA).

3.1.3 Effects of Early Decontamination of HD on Lesion Size Estimates

Previous work at the MREF in Task 85-11 with MREF Protocol 22 showed a significant ($P < 0.05$) animal-to-animal variation in lesion size response. For this reason, unilateral estimates from lesions decontaminated with M258A1 I and II in these studies could not be directly compared with similar estimates from a control study. However, the approval of an early decontamination step depends on there being no loss of sensitivity to contralateral differences in

the experimental model. Therefore, comparisons could be made between contralateral differences in the MREF Protocol 22 validation studies and those in early decontamination studies in Optimization A.

The initial MREF Protocol 22 validation attempt in Task 85-11 with HD decontaminated at 1.25, 5.0, and 10.0 min after dosing was not successful in demonstrating a significant difference between distilled water and M258A1 I and II at each time. At the time of these Optimization A studies, the shorter times at which MREF Protocol 22 was later validated (1.0, 3.0, and 5.0 min) had not yet been determined. Nonetheless, the comparison of contralateral analyses at 1.25, 5.0, and 10.0 min was considered an accurate index of loss or gain in sensitivity of the model to evaluate the effects of the 4-hr NaClO decontamination.

Tables 3.1.2 to 3.1.4 present data from the decontamination with 0.5 percent NaClO at 4-hr after dosing. Tables 3.1.5 to 3.1.7 present individual lesion size data from the MREF Protocol 22 initial validation attempt for HD, using 5.0 percent NaClO at 24 hr after dosing. Table 3.1.8 presents summary statistics for both studies, including mean lesion sizes that are plotted in Figures 3.1.1 through 3.1.6. Contralateral mean differences and the significance of those differences are presented in Table 3.1.9.

Analysis of results from the early HD decontamination study showed a statistically significant ($P < 0.05$) contralateral difference between the M258A1 standard and distilled water decontamination systems for all times and for the overall average difference. The differences were significant for lesion lengths, widths, and areas. Analysis of the 24-hr HD decontamination data (MREF Protocol 22) showed significant differences at all times except at 10 min for lesion lengths, widths, and areas (this was why MREF Protocol 22 was not validated for HD at 1.25, 5.0, and 10.0 min, since the differences had to be significant at every time for a validation).

Further comparison of these studies is made in Table 3.1.10, which demonstrates the effects of the earlier decontamination of HD. In Table 3.1.10, "Difference" is the contralateral difference for the 24-hr decontamination of HD with 5.0 percent NaClO less the contralateral difference for the 4-hr decontamination with 0.5 percent NaClO. Thus, "Difference" is an absolute measure of the increase in effectiveness of M258A1 I and II relative

to distilled water in removing and decontaminating the HD dose 20 hr earlier. Positive "Difference" implies improved relative effectiveness by the M258A1 standard. The t statistic is the studentized "Difference" represented in standard error units and is statistically significant ($P < 0.05$, two-sided) when greater than 2.02.

The lesion length contrasts showed a significant improvement for 5.0 and 10.0 min in the HD model, with a 4-hr NaClO decontamination following M258A1 decontamination. As a direct result, the overall average contralateral difference was also significantly increased. These improvements were due to the enhanced effectiveness of the M258A1 system when followed 4 hr later by the 0.5 percent NaClO solution, rather than to any shift in the effectiveness of the water between studies. Decrements in the average contralateral differences were evident at 1.25 min for lesion lengths and widths (and therefore areas), but these were not significant.

The results obtained after performing the HD decontamination/removal process with a nonirritative 0.5 percent NaClO solution, followed by two distilled water rinses at 4 hr after dosing, demonstrated that a significant change had been made in the experimental model. The change resulted in an apparent improvement in the model's sensitivity in distinguishing between an inadequate HD decontamination system (distilled water) and the standard dual components of the M258A1 kit.

Therefore, the 4-hr decontamination process did not decrease the sensitivity of the model nor did it reduce the effectiveness of the model in discriminating between an effective and noneffective decontamination system. Thus, it appears warranted to remove animals from the hoods after a 4-hr decontamination with 0.5 percent NaClO.

3.1.4 Effects of Early Decontamination of L on Lesion Size Estimates

Lesion lengths, widths, and areas for the decontamination of L with 0.5 percent NaClO at 4 hr after dose are presented in Tables 3.1.11 to 3.1.13, respectively. Similar data obtained in Task 85-11 from the attempted validation of MREF Protocol 22 for L decontaminated with either M258A1 I and II or distilled water followed by 5 percent NaClO solution at 24 hr are presented in Tables 3.1.14 to 3.1.16. Average lesion sizes are presented for both studies in Table 3.1.17 and are plotted in Figures 3.1.7 through 3.1.12. Comparison of contralateral differences between these studies is made in Table 3.1.18.

The MREF Protocol 22 validation attempt in Task 85-11 failed to demonstrate a significant difference between contralateral lesion lengths for L decontaminated with M258A1 I and II versus distilled water used twice. The contralateral difference in lesion length was significant ($P < 0.05$) at 30 sec after dosing to decontamination but not at 60 or 120 sec. When L decontamination and removal were performed with a nonirritative 0.5 percent NaClO solution 20 hr earlier, the contralateral differences in lesion length were statistically significant ($P < 0.05$) at all three times.

Further comparison of these studies is made in Table 3.1.19, which presents the effects of the early decontamination of L. In Table 3.1.19, "Difference" is the contralateral difference for the 24-hr decontamination of L with 5.0 percent NaClO less the contralateral difference for the 4-hr decontamination with 0.5 percent NaClO. Thus, "Difference" is an absolute measure of the increase in relative effectiveness of M258A1 I and II in removing and decontaminating the L dose 20 hr earlier. Positive "Difference" implies improved relative effectiveness by the M258A1 standard. The t statistic is the studentized "Difference" expressed in standard error units and is statistically significant ($P < 0.05$, two-sided) when greater than 2.02.

The lesion length contrasts showed a significant ($P < 0.05$) improvement for all times in the L model, with a 4-hr NaClO decontamination following M258A1 decontamination. Changes for lesion widths and area were all positive, therefore increasing the sensitivity of the model, but these changes were not statistically significant.

When the L decontamination/removal process is performed with a nonirritative 0.5 percent NaClO solution, followed by three distilled water rinses at 4 hr after dosing, the results demonstrate a significant change in the experimental model. The changes are an apparent improvement in the model's sensitivity in distinguishing between an inadequate L decontamination system (distilled water) and the standard dual components of the M258A1 system. Therefore, the 4-hr decontamination process does not reduce the sensitivity or effectiveness of the model in discriminating between effective and noneffective decontamination systems. Thus, it appears warranted to remove animals from the hoods after a 4-hr decontamination with 0.5 percent NaClO.

3.2 OPTIMIZATION B

Results of Optimization B studies are presented in the following sections. Data from studies of positional and side effects are given in Tables 3.2.1 through 3.2.13 for HD and in Tables 3.2.14 through 3.2.21 for L. Figures 3.2.1 through 3.2.6 show the data for HD studies and Figures 3.2.7 through 3.2.9 plot the data from L studies.

3.2.1 Position, Side, and Time Effects for HD

HD lesion size estimates (lengths, widths, and calculated areas) are presented by dose group in Tables 3.2.1 through 3.2.4 for the study performed with the original (nonvalidated) times to decontamination of 1.25, 5.0, and 10.0 min and 24 hr. Tables 3.2.5 through 3.2.8 present similar data for the study performed with the MREF Protocol 22 validated times of 1.0, 3.0, and 5.0 min and 24 hr. Mean (n = 8) lesion estimates averaged by position, side, and time are presented for these studies in Tables 3.2.9 and 3.2.10. These means are plotted by lesion response in Figures 3.2.1 through 3.2.3 for the original times and in Figures 3.2.4 through 3.2.6 for the validated times. To emphasize position and side effects, the data are presented as averages (n = 32) across times by position and side in Table 3.2.11, which also presents marginal means by position (n = 64) and by side (n = 128), as well as the overall estimated mean (n = 256) length, width, and area per study.

Selected effects involving the factors position, side, and time from the ANOVA are presented for the HD studies in Table 3.2.12. Significance levels for Type III sums of squares indicated a significant ($P < 0.001$) effect of time to decontamination on lesion length, width, and area in both HD studies. This was expected, since the experimental model had been designed to compare the effectiveness of decontamination systems at times that resulted in distinguishable lesion sizes. Side was a significant factor ($P < 0.05$), both for the lesion length response in the original study and for lesion width in the study using the validated times to decontamination. Position was a significant factor ($P < 0.01$) for lesion widths in the original study and for lesion lengths in the latter study. Area was not significantly affected by position or side in either study. The interaction of position with time was significant ($P < 0.05$) for width and area in the original study. All other interactions were nonsignificant ($P \geq 0.05$) for all lesion size responses in both studies.

Results from the tests of trends and two-sample contrasts are presented in Table 3.2.13. Significance of the time effect shown by the ANOVA was demonstrated in the trend tests as linearity of estimates averaged over all positions and both sides with time, which was significant ($P < 0.001$) for all lesion responses in both HD studies. Another significant trend in the original HD study was lesion widths averaged over sides and times having linear ($P < 0.05$) and quadratic ($P < 0.01$) effects with position. That is, HD lesion widths were smaller in the middle of the rabbit back than at either end. Areas also exhibited significant ($P < 0.05$) linear trends with position in the original HD study.

A quadratic relationship ($P < 0.01$) existed for lesion lengths with position in the latter HD study, which reiterates the positional effect mentioned earlier. That is, in the latter HD study, lesions were significantly longer at the middle two positions on the rabbit back than at the ends.

Results of the contrast tests showed more specifically the sources of the side effects previously identified by the ANOVA results. In the original HD study, side had a significant ($P < 0.05$) effect on lesion lengths due to the contralateral differences, especially at positions 3 and 4 at 5.0 min. These were the more difficult positions to reach for dosing and

decontamination by technicians. In the latter HD study, side had a significant ($P < 0.05$) effect on lesion widths due to the contralateral differences, especially at decontamination 3 min after exposure. Since these effects were sporadic and not common across studies for any lesion response, and since a Type I error (rejecting a true null hypothesis) is expected in 5 percent of these contrasts, the significance attributed them must be regarded with some reservation.

3.2.2 Position, Side, and Time Effects for L

L lesion size estimates (lengths, widths, and calculated areas) are presented by dose group in Tables 3.2.14 through 3.2.17 for the study performed with times to decontamination of 30, 60, and 120 sec and 24 hr. Mean ($n = 8$) lesion estimates averaged by position, side, and time are presented for these studies in Table 3.2.18. These means are plotted by lesion response in Figures 3.2.7 through 3.2.9. To emphasize position and side effects, the data are presented as averages ($n = 32$) across times by position and side in Table 3.2.19, which also presents marginal means by position ($n = 64$) and by side ($n = 128$), as well as the overall estimated mean ($n = 256$) length, width, and area for the study.

Results from the ANOVA are presented for the L study in Table 3.2.20. Significance levels for Type III sums of squares indicated a significant ($P < 0.001$) effect of time to decontamination as prescribed by the experimental model. Anterior-posterior position and the interaction of position with time were also significant ($P < 0.001$) for lesion widths and areas, but not for lengths. That is, lesion widths were shorter at the middle of the rabbit back than at the ends, but lesion lengths were statistically unchanged ($P = 0.1447$), thereby resulting in smaller lesion areas at the middle of the back than at the ends. All other interactions were nonsignificant ($P \geq 0.05$).

Results of the trend analyses and two-sample contrasts, presented for L in Table 3.2.21, agreed with the ANOVA. There was significant ($P < 0.001$) linear fit of all lesion responses averaged over all positions and sides with time to decontamination. Estimates averaged over both sides and all times had significant quadratic trends with position; lesion lengths had

significant quadratic trends at $P < 0.05$ (not detected in the ANOVA), and lesion widths and areas had significant quadratic trends at $P < 0.001$. Each of these estimates was smaller in the middle of the rabbits' backs than at the ends. All contralateral contrasts were nonsignificant ($P \geq 0.05$).

3.3 OPTIMIZATION C

3.3.1 Effects of M258A1 I and II Packaging (Bulk Versus Kit) Against HD

Lesion size estimates (lengths, widths, and calculated ellipsoid areas) are presented in Tables 3.3.1 through 3.3.3, respectively, for 20 animals dosed with HD and decontaminated with M258A1 components I and II from either bulk preparations or field kit foil packets. The same data are summarized in Table 3.3.4 and Figures 3.3.1 through 3.3.3, which present the lesion size mean estimates at each time to decontamination for both packaging forms. One-sided contrasts showed statistical equivalence between bulk and kit packaging at every time to decontamination for lesion lengths, widths, and calculated areas. Thus, whether a HD lesion was decontaminated with M258A1 components I and II from bulk preparation or from field kit foil packets, there was no significant difference in the size of the lesion.

3.3.2 Effects of M258A1 I and II Packaging (Bulk Versus Kit) Against L

Lesion size estimates (lengths, widths, and calculated ellipsoid areas) are presented in Tables 3.3.5 through 3.3.7, respectively, for 20 animals dosed with L and decontaminated with M258A1 components I and II from either bulk preparations or field foil kit packets. Due to technical error, four animals were not dosed at the site to be decontaminated at 24 hr on the M258A1 kit (left) side, and lesion size estimates were not collected. However, statistical analyses were performed on data at 24 hr from the other 16 animals. The data are summarized in Table 3.3.8 and Figures 3.3.4 through 3.3.6, which present the lesion size mean estimates at each time to decontamination for both packaging forms.

One-sided contrasts showed a significantly greater ($P < 0.001$) mean lesion length at the 24-hr decontamination on the field kit (left) side relative to the bulk (right) side. These sites were theoretically dosed and decontaminated identically. In view of the demonstration of no side effect in Optimization B, the most plausible explanation for the difference is that the left side dose was systematically applied over a greater distance. The most posterior site on the animal's left side is the most difficult site for a right-handed technician to reach and dose with agent consistently. The observed discrepancy between the lesion lengths at the two 24-hr positive control decontamination sites places doubt on the remaining results of this study for L, since we cannot be certain that a similar error in dosing was not present at other times to decontamination.

Table 3.3.8 also shows that there was a significantly greater ($P < 0.05$, one-sided) mean lesion width for M258A1 field kit components used at 120 sec but a significantly smaller mean width at 24 hr relative to the M258A1 bulk components. There were no significant differences in lesion areas due to M258A1 packaging at any time to decontamination.

3.4 OPTIMIZATION D

3.4.1 Results from Optimization D Studies

Lesion length estimates are presented in Table 3.4.1 for 16 animals dosed with HD on six sites and decontaminated with M258A1 I and II on the left side at either 20, 45, and 75 sec or at 10, 15, and 60 sec after dosing. Both sides were decontaminated at 24 hr after dosing with a 5 percent NaClO solution followed by three distilled water rinses. Lesion length ratios (left/right) are presented in Table 3.4.2, in which outliers are indicated by an asterisk. The data in Table 3.4.2 describe a growth curve for lesion length ratios from 10 to 75 sec after dosing.

3.4.2 Results from MREF Protocol 22 Validation Studies

For information about the curve beyond 75 sec, we had already obtained similar data under MREF Protocol 22 validation work. In those studies, the right side of each animal was dosed at four sites. The three anterior sites were decontaminated with M285A1 I and II either at 75, 300, and 600 sec (24 animals) or at 60, 180, and 300 sec (24 animals). The most posterior site was a control. At 24 hr, all four sites were decontaminated with a 5 percent NaClO solution followed by three distilled water rinses.

The validation model thus differed from Optimization D in that the 24-hr control lesion was not contralateral but was instead at a site that had previously been shown to affect the size of the lesion. However, the magnitude of the effect for each anterior-posterior position was known from Optimization B, as shown in Table 3.4.3 for HD lesion lengths. Lesion lengths were pooled across times to decontamination for calculating mean lesion lengths (n = 64, nominally) by position. Data on Tables 3.2.1 through 3.2.4 were used to correct validation data at 1.25, 5.0, and 10 min. Data in Tables 3.2.5 through 3.2.8 were used to correct validation data at 1, 3, and 5 min. The correction terms given in Table 3.4.3 were subtracted from the appropriate validation data presented in Tables 3.4.4 and 3.4.5. Position-corrected data are expressed as a fraction of the 24-hr control lesion length for each animal in Tables 3.4.6 and 3.4.7, in which outliers are indicated by an asterisk.

3.4.3 Regression Analyses

The ratios ($R_L = L_t/L_{24\ hr}$) from MREF Protocol 22 validation work were combined with the ratios from Optimization D work in the generation of a weighted least-squares regression curve of the form

$$R_L = 1 - B_2[e(-B1t)] \quad ,$$

where

R_L = ratio of lengths,

L_t = lesion length at 24 hr after decontamination at time t,

$L_{24\ hr}$ = lesion length at 24 hr after decontamination at 24 hr,

e = Naperian base, 2.71828...,

B_1 = exponential regression parameter, and

B_2 = fractional compliment to the y-axis intercept.

Solutions found for B_1 and B_2 were 0.003108 and 0.51009, respectively. The data are plotted with the regression curve and 95 percent confidence limits about the mean in Figure 3.4.1. Since B_2 was 0.51009, the y-intercept in the plot was $1 - B_2 = 0.49$, which was greater than the target lesion length ratios of 0.25. The objective of this optimization was to determine the three times to decontamination at which HD lesion size reached 25, 50, and 75 percent of the maximum. Since this was not possible with the response $R_L = L_t/L_{24\ hr}$, we performed a nonlinear regression analysis on lesion growth ratios, defined as $R_G = (L_t - 10\ mm)/(L_{24\ hr} - 10\ mm)$, in which the original dosing contribution (10 mm) to the lesion was subtracted to provide the true growth of the lesion after application. MREF Protocol 22 validation data (corrected for position effects) were included. Lesion growth ratios are presented in Table 3.4.8. The model was

$$R_G = 1 - B_2 e^{(-B_1 t)} \quad ,$$

in which the curve was forced through the origin, and the solutions for B_1 and B_2 were 0.003561 and 0.8113, respectively.

The data are plotted with the regression curve and 95 percent confidence limits about the mean in Figure 3.4.2. Solving for the t value in the lesion growth ratio equation gave

$$t = \frac{\ln\left(\frac{1-R_G}{B_2}\right)}{-B_1} \quad .$$

Values for t at specified R_G values are presented in Table 3.4.9. The times to decontamination that were predicted to yield lesion length growth ratios of 25, 50, and 75 percent were approximately 30, 150, and 330 sec (or 0.5, 2.5, and 5.5 min). The minimal lesion growth ratio was approximately 0.20. Theoretically, the curve should have included the origin, if M258A1 were a perfect HD decontamination system. The regression suggested that in an average HD lesion, growth could not be arrested more than 80 percent by M258A1 I and II decontamination, regardless of how quickly decontamination followed dosing.

3.5 OPTIMIZATION E

Six optical technologies were evaluated in an attempt to find a suitable replacement for the Draize scoring system for noninvasively assessing skin irritation. The technologies included automated image analysis, thermography, laser doppler velocimetry, photopulse plethysmography, and reflectance photometry. We also developed and tested a binomial response method for visually comparing dermal irritation at contralateral HD lesion sites.

In addition, automated image analysis and manual planimetry were evaluated for how well they could be used to estimate lesion sizes. The current method for estimating lesion sizes in the MREF Protocol 22 screen is to measure the lesion length and width with a ruler. Pass or fail screen results are based on paired t tests using lesion length as the primary response, and lesion width and calculated (ellipsoid) area as corroborative responses.

The problem with this procedure is that lesion length is not only a function of how well the decontamination system prevents lesion length extension, it is also a function of the accuracy of manually applying 0.5 µl of HD over a 1-cm length. Similarly, lesion width is related to how evenly the dose is placed and to the length of application. Lesion area would appear to be the best response, since it is affected less by inaccuracies in dose application than are lesion length and width.

Most HD lesions generated under MREF Protocol 22 are ellipsoid in shape. However, our experience has shown that they are occasionally pear or dumbbell shaped. For such lesions, the calculation of area as 0.25π times the product of length and width (assuming an ellipsoid) results in an

overestimation. An area integrator is needed. Thus, we tested the image-sizing features of an automated image analyzer and of a manually operated planimetry system developed for an IBM personal computer.

Our scheme for evaluating each technology consisted of three or four phases. The first phase was our familiarization with the principles of operation, the response(s) measured and the assumptions implicit in interpreting the response(s), and a review of the pertinent literature. The second phase included the selection and acquisition of an instrument for evaluation and our familiarization with its operation. Then using the non-irritated skin of our hands and forearms, we investigated the instrument's sensitivity to different operating conditions, which we tried to optimize.

The third phase was a crude evaluation of the instrument's ability to measure dermal irritation in the MREF Protocol 22 rabbit model on a limited scale. Our samples for analysis were three rabbit backs dosed with one of three irritants, i.e., a 10 percent potassium hydroxide solution, a household disinfectant, or a household ammonia cleaner. Our intent in using these irritants as mock chemical warfare (CW) vesicants was to preclude the possibility of contact between CSM and an instrument on loan to us from the manufacturer.

The irritants were applied over a range of dose volumes such that resultant lesions would resemble HD lesions in shape over a range of sizes and in degrees of erythema and edema. Dose volumes for each irritant ranged from 0.10 to 0.75 ml and were applied unilaterally (right side only) on clipped rabbit dorsa previously marked with the MREF Protocol 22 dosing regimen outline. The rabbits were anesthetized with ketamine (8.75 mg/kg) and xylazine (2.5 mg/kg) previous to irritant dosing.

Immediately after irritant dosing, the dose sites were covered with gauze, and the animals were girded with Vetwrap to prevent evaporation of the irritants. At 2 hr after dosing, the rabbits were unwrapped and anesthetized as before for evaluation of irritation at the dose sites. Each instrument was subjectively evaluated for its apparent ability to confirm visually evident dermal irritation. Efficacy judgement was based on the value of the response relative to the price of the instrument and its cost of operation in time and the number of operators required.

The fourth phase was performed for any technique that passed the crude test just described. In the fourth phase, the technique was evaluated using 24 rabbits in a MREF Protocol 22 validation. The instrument was assessed for its ability to detect differences in irritation between sites dosed with 0.5 µl of HD followed by decontamination with either distilled water or M258A1 I and II.

3.5.1 Automated Image Analysis

Software advances in digital picture processing, coupled with increased microprocessor speeds, have made possible a relatively new technology that has been loosely labeled image analysis. Some of the major microscope manufacturers have marketed microcomputer-based systems that attempt to meet the need for image analysis in histomorphometry and stereology. In general, these instruments operate by collecting a live video image on a monitor, digitizing the image, stepping through an operator-defined image-enhancement program, detecting desired objects versus background, measuring or counting those objects, and saving the data and the image on a magnetic disk.

We evaluated one of the most versatile of the commercially available instruments, the Magiscan 2, which is manufactured by Joyce-Loebl LTD (Gateshead, U.K.) and distributed by Nikon, Inc. (Garden City, NY). Our evaluation of Magiscan 2 was based on its ability to distinguish areas of irritated dermal skin from normal skin and to accurately quantify the size and intensity of irritation (integrated grey-level density) of each lesion. The Magiscan 2 consisted of a photomultiplier video camera, a high resolution color monitor, and a desk-top microprocessor with keyboard, light pen, and dual disk drives. The video camera was readily adapted for imaging lesions on backs of rabbits by fitting it with a 55-mm f2.8 Micro-Nikkor lens.

The video camera was mounted on a camera support with a vertical adjustment. In addition to fluorescent office lighting, the camera field of view was illuminated with a fluorescent desk lamp with dual 15-watt tubes. The Magiscan 2 was able to distinguish 100 grey levels between black and white. We calibrated the instrument with the image of a plastic ruler while using a calibration program resident in the system software. One rabbit at a

time was positioned under the camera, and adjustments of the camera's vertical position and lens were made so that the monitor screen was filled with the area of dorsal skin containing all lesions. We attempted to differentiate the lesions from normal skin on the basis of grey levels. A green filter was included in the camera lens assembly to selectively darken erythema in the image.

We found that the boundary between lesion and normal skin was too diffuse for the instrument to consistently match what was apparent by visual examination and measurement by ruler. That is, the lesion/normal skin contrast involved too few grey levels for the operator to define a grey level threshold that would constitute a reliable benchmark for the instrument (such a threshold was necessary for the instrument to automatically distinguish lesions from normal skin on a given rabbit). This was true for the potassium hydroxide solution and the disinfectant. Irritation from the ammonia solution was not apparent either visually or by means of the image analyzer.

The curvature of the rabbit back greatly contributed to problems in obtaining uniform lighting of the field of view. Lighting gradients, however slight, were enough to create artifact in the imaging of lesions. We were impressed by the superiority of the human eye over the image analyzer in perceiving slight variations in color and light intensities and the advantage to image processing afforded by experience in knowing what lesion shapes to expect.

It was surmised that even if lighting gradients could be eliminated, the inter-rabbit variation in normal skin grey levels would preclude the generation of an all-purpose lesion evaluation program. An operator would have to set the lesion grey level threshold for each rabbit prior to analysis. The time spent interactively by an operator in setting that threshold would be better spent by deciding for himself what the lesion boundaries were and by using the light pen to trace them on the monitor. We felt that the labor intensity of that evaluation scheme did not justify the price of the Magiscan 2, which is approximately $70,000. We concluded that the Magiscan 2 was not a cost-effective solution to measuring HD skin irritation and lesion sizes. Instead of investigating other automated image analysis systems, e.g., the IBAS by Zeiss and the TAS PLUS by Leitz, we decided to look at the efficacies of other optical technologies in quantifying skin irritation.

3.5.2 Thermography

Thermography measures the infrared radiation emitted by every object with a temperature above absolute zero. The relationship between irradiated infrared light and surface temperature is dependent upon physical characteristics (emissivity) of the material. Fortunately, skin is a near-perfect emitter so that the amount of infrared energy detected over a given area is an index of skin surface temperature.

We tested an Inframetrics (Bedford, MA) Model 525 Imaging Radiometer System for its ability to assess skin surface temperature as an index of irritation from topical exposure to one of three irritants. Three animals were used as described in Section 3.5, except that a commercial depilatory was used instead of the ammonia solution. Our intent was to determine whether skin surface temperature consistently increased or decreased with the degree of irritation resulting from contact with chemicals.

The Model 525 was comprised of a tripod-held scanner, control unit, portable power supply, video cassette recorder, colorizer, and color video monitor on a portable cart. The scanner contained a mercury/cadmium/tellurium detector cooled by liquid nitrogen. The instrument had a temperature measurement range of -20 to 1300 C in the 8 to 12-um spectral region, with a minimum detectable temperature difference (thermal resolution) of 0.1 C at 30 C ambient temperature. Other specifications included a flicker-free, real-time video display of 30,000 picture elements per frame.

The colorizer automatically assigned color codes to thermal steps defined by the operator. Thus, surface temperatures were indicated within an operator-selected range as a spectrum from blue (coldest) to red (hottest). The range was selected on the control unit. By "freezing" an image on the video display and by switching to the line scan mode, a thermal profile could be displayed for points along any straight line positioned through the image by the operator. Temperatures of the lesion surface relative to normal skin were thus displayed graphically, and thermal relationships were readily quantified.

Orienting the scanner so that it was perpendicular to the rabbit dorsum was complicated by the requirement that the scanner's detector remain

in contact with liquid nitrogen stored above it in a small Dewar flask. Tipping the scanner too far from horizontal temporarily suspended the system's operation. Our solution was to turn the rabbit partially onto its side and assess only one side of lesions at a time. For samples as small as the lesions in our model, the instrument was intended to be fitted with a closeup lens, which was not available to us. Without it, we were barely able to fill the video monitor screen with one dose site while scanning from the instrument's minimum focal distance of approximately 15 cm.

We tried scanning at the maximal thermal resolution of 0.1 C, but the rabbit skin surface temperature was too unstable to render a clear image on the monitor. That is, individual picture elements changed colors too rapidly for us to see a clear lesion boundary. At a thermal resolution of 0.25 C, we obtained a more stable image, but the difference between the warmest part of the lesion and normal skin was only 0.75 C. In addition, the monitor image did not reliably corroborate what was visually observed as irritated tissue. In the center of some lesions was an area of cooler tissue, presumably caused either by focal loss of circulation or by focal fluid evaporation.

Hackett (1974)[4] has demonstrated the utility of thermography in assessing the degree of burn based on temperature drop (up to 2.5 C) associated with destruction of blood vessels in burn patients. In our test, the centers of some lesions had temperatures equal to or only 0.25 C below that of normal skin. In most lesions that we examined, the thermogram appeared as an oval ring of lesion boundary (warmer than normal skin) surrounding a cooler lesion center. However, in some thermograms, the ring was not complete, and the lesion center was not discernible from normal skin, which resulted in a cup-shaped pattern. Assuming evaporative cooling in the lesion centers, we tried covering the rabbit dorsum with a thin plastic film. The result was a more homogeneous lesion thermogram, but the lesion boundary became less resolved due to the insulating effect of the film.

We concluded that a maximum thermal difference between lesion and normal skin of 0.75 C (three resolvable 0.25 C steps) was not a wide enough scale to attempt quantifying relative irritation from lesion to lesion. The scale may have been wider using a severe vesicant, such as HD or L, but we

were reluctant to take a loaned instrument into the MREF dosing laboratories. In summary, the Inframetrics Model 525 Imaging Radiometer System successfully imaged the lesions as stable thermograms, but we believe that the thermal difference between normal and irritated skin was not enough to use skin surface temperature as an index of irritation for comparing lesions.

3.5.3 Laser Doppler Velocimetry (LDV)

In LDV, a helium-neon laser beam irradiates skin to a depth of 1 to 1.5 mm. The light is back scattered by stationary tissue and moving blood cells in the microvasculature. The back-scattered light, which is Doppler-shifted toward lower frequencies by an amount relative to the velocity of the moving cells, is collected by a photometer. The resultant frequency shift profile is converted into a mean blood flow rate parameter and is measured in millivolts. Faster blood flow renders a greater voltage output. LDV has been linearly correlated ($P < 0.001$) with 133-xenon clearance as a valid blood flow rate parameter (Holloway and Watkins 1977[5] and Stern et al. 1977)[8].

Our intent in evaluating LDV was to determine whether peripheral blood flow rate in rabbit dorsal skin was a good index of skin irritation from chemical burning. We obtained a model LD5000 Laser Doppler Capillary Perfusion Monitor (Medpacific, Seattle, WA) and used it with the three-rabbit model described in Section 3.5. The instrument consisted of a 5-mW HeNe laser beam delivered through an optical fiber to a 1.9-cm diameter probe, which was held on the animal's back with finger pressure. Reflected light, both Doppler-shifted and nonshifted, was returned by another optical fiber to the instrument. The relative flow rate was expressed in millivolts on a light-emitting diode display and on a strip recorder.

The LD5000 was simple to use, gave immediate results, and responded rapidly to changes in blood flow. When the probe was held to a human finger tip and manual occlusion was applied at the finger's first knuckle, the LD5000 output dropped from approximately 30 mV to zero within a sec. When occlusion was ceased after 6 sec, the LD5000 output indicated resumed blood flow within 0.2 sec, and fully normal flow within 10 sec. Blood flow seemed to be site-dependent; the output varied from 30 to 52 mV over four fingertips of one

human hand. We found the instrument to be so sensitive to blood flow rate that its sensitivity had to be attenuated in order to smooth out pulsatile deflections, including a dichrotic notch associated with heart beats.

When we tested the instrument on rabbits, we again observed site-dependent output variability. For example, three nonirritated control sites on one rabbit gave outputs of 20, 28, and 33 mV. Contralateral lesions gave outputs of 31, 40, and 37 mV, respectively. On another animal, control sites gave 43, 27, and 41 mV and contralateral lesions gave 34, 60, and 32 mV, respectively. By visual examination, each of the lesions was more erythemic than any one of the control sites. Yet there seemed to be no correlation of LD5000 output voltage with erythema.

A contralaterally paired t test (two-sided, alpha = 0.05) between control and lesion site outputs showed no statistically significant difference ($P = 0.326$) for the animals examined. There may have been an artifact problem in the above readings due to the hair stubble remaining on the clipped rabbit dorsa. Since stubble prevented the probe from contacting the epidermis, we suspected that the probe was either not irradiating enough skin or not collecting the proper Doppler-shifted light signal. MREF Protocol 22 called for the use of clipped but not depilated rabbit skin to screen HD decontamination systems. Thus, we did not pursue the possible use of the LD5000 on depilated rabbit skin or rabbit ears.

In summary, LDV as represented by the Medpacific LD5000 appeared to be sensitive to blood flow rate at a given reading site. However, flow readings from site to site appeared to vary considerably, probably due to the difference in the microvasculature field beds at each site, but possibly due to light interference from hair stubble. There was no discernible difference between normal skin and chemically irritated skin as measured by the LD5000 instrument.

The results may have been different using HD-dosed animals. However, the instrument had been made available on a loan basis, and there was reluctance to touch the probe to CSM-dosed skin. It was felt that the results of the preliminary investigation did not warrant purchase ($14,000) of an LD5000 for further testing.

3.5.4 Photopulse (or Photoelectric) Plethysmography (PPG)

In PPG, infrared light from a light-emitting diode (LED) resident in a small probe enters the skin to an approximate depth of 1 cm. The wavelength of the emitted light is at the isosbestic point for hemoglobin and oxyhemoglobin, 800 nm. The light is therefore equally absorbed by arterial and venous blood, and the unabsorbed light is measured by a photodetector positioned in the probe adjacent to the LED. The signal is sent to an amplifier and is either displayed in toto or split into a pulsatile component and a total blood volume component. The height of the pulsatile component may be interpreted as an index of blood flow. The total blood volume component is a volumetric response that increases with vasodilatation, e.g., due to mechanically restricted venous return.

The intent in evaluating PPG was to determine whether total blood volume within a local region of skin might be an index of dermal irritation in laboratory rabbits. A photopulse plethysmometer (no model number) from Medasonic (Mountain View, CA) was obtained and used with the three-rabbit model described in Section 3.5. Voltage output was indicated digitally and by a strip chart recorder.

The instrument was determined to be unsuitable for this application. Control sites rendered widely varied (total blood volume) voltage output, even when limited to a single rabbit. This was interpreted to be reflective of the instrument's sensitivity to changes in the microvascular field bed from site to site, as previously suggested by Challoner (1979)[1]. Even when the probe was kept at one site, the voltage output could be modified by changing the force used to hold the probe against the rabbit's skin. Pressing the probe onto the skin with more force caused the output to drop, apparently due to local skin blanching. The output also decreased if the probe was not held down onto the skin, i.e., if it was allowed to rest on the hair stubble of the clipped rabbit. Also, tne effect of ambient light upon the instrument's voltage output required us to work either in subdued lighting or with a dark cloth over the probe. It was felt both of these restrictions were unacceptable. The variation contributed by these factors totally masked any blood volume effect associated with irritation.

With the probe taped to a rabbit ear and covered with a dark cloth, we were able to demonstrate peripheral vasoconstriction associated with sodium pentobarbital anesthesia (5 mg/kg). The voltage output dropped gradually over a 5-min period following administration of anesthetic and remained at approximately two-thirds of the normal level for at least 10 min.

In summary, the photopulse plethysmometer appeared to be a sensitive indicator of local blood volume, but was too site-dependent and too sensitive to operating conditions to be used in obtaining irritation scores in our MREF Protocol 22 screen.

3.5.5 Reflectance Photometry

Reflective photometry is used in manufacturing for quality control of appearance (i.e., brightness, opacity, gloss, and color) in the production of paper, plastics, cosmetics, and paints. An incandescent white light is passed through an appropriate color filter and the monochromatic light is emitted from a probe held to the surface of a sample. The filter color is selected to complement the sample color, i.e., so that the emitted light is maximally absorbed by the sample surface. The reflected light is detected by a photometer in the probe, and percent reflectance is displayed digitally by the control unit. Standards are used to calibrate the instrument.

A model 575 Reflectance and Gloss Meter with a Y-type search unit (probe) was obtained from Photovolt Corporation (Indianapolis, IN). This instrument was used to determine whether the intensity of reflected light from lesions might be used as an index of dermal irritation induced by topical application of chemicals.

The three-rabbit model described in Section 3.5 was initially used, except that a 10 percent NaClO solution was used instead of the household ammonia solution. Also, the irritants were applied bilaterally instead of only on the right side. In the initial test, a color filter was not used. The instrument was calibrated to a zero reflectance standard and scaled so that each animal's normal skin served as its own control. That is, the instrument was gained for each animal so that normal skin gave approximately 100 percent reflectance of white light. The probe had a 2-cm diameter

(~310 mm^2 area) aperture that was manually centered at the darkest part of each lesion for reading.

It was found that the 10 percent NaClO lesions were too small (all lesion areas were between 30 and 150 mm^2) to be represented by readings of areas that included at least as much normal tissue as irritated tissue. Also, when the household cleaner was rinsed off the backs prior to reading, the felt tip ink was smeared onto the dose sites. It was felt that the resulting reflectance was probably lowered by the presence of the ink smears. The following table contains the results of the 10 percent potassium hydroxide lesions produced on one rabbit. The probe aperture generally encompassed more than the width of each lesion but less than its length.

PERCENT REFLECTANCE OF 10% KOH LESIONS
MEASURED BY PHOTOVOLT MODEL 575

Dose Volume (ml)	Length of Dose Application (cm)	Percent Reflectance	
		Left Side	Right Side
0.10	0.50	87	89
0.25	1.0	85	77
0.50	1.5	71	85
0.75	2.0	65	71

It was felt that the inverse relationship between dose volume and percent reflectance as indicated above was due more to the area of lesion involvement than to the intensity of erythema.

Twenty-four animals dosed with HD and decontaminated at 0.75, 1.5, and 3.5 min were scored by the Draize method at 24 hr after dosing. Scoring was guided by the following:

Erythema Formation

No erythema	0
Very slight erythema (barely perceptible)	1
Well-defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation (injuries in depth)	4
Highest possible erythema score	4

Edema Formation

No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edges of area well-defined by definite raising)	2
Moderate edema (raised 1 mm but not extending beyond area of exposure)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Highest possible edema score	4
Highest possible total score	8

Descriptive words were correlated with values of the primary irritation index as follows:

0-2 = Mildly irritating
2-5 = Moderately irritating
5-6 = Moderately to severely irritating
6-8 = Severely irritating.

The same lesions were read by the reflective photometer fitted with an in-house 6-mm aperture. Except for that aperture, the probe was protected from HD contamination with a plastic covering. A color filter was not used. The control site that received no HD dose was used as each animal's control site for calibrating the instrument to 100 percent reflectance.

Results of the Draize scoring and reflectance scoring are presented for each animal in Tables 3.5.1 and 3.5.2, respectively. Results of t test analyses of contralaterally paired Draize scores (erythema, edema, and total irritation) and reflectance scores are presented in Table 3.5.3. Draize erythema, edema, and total irritation scores were significantly greater (alpha = 0.05, two-sided) for distilled water than for M258A1 I and II at all times to decontamination. Reflectance score means were significantly ($P < 0.05$) lower, indicating darker (i.e., presumably more erythemic) lesions for distilled water than for M258A1 I and II at 0.75 and 1.5 min to decontamination. The reflectance score means were not significantly different ($P = 0.6022$) at 3.5 min to decontamination. Reflectance scores seemed to be too similar for distilled water versus M258A1 to infer any biologic significance to the differences.

In summary, the reflectance meter needs to be examined more closely under optimal operating conditions and with color filtration. Preliminary results have shown statistical differences between decontamination with distilled water versus the M258A1 I and II standard system.

3.5.6 Binomial Irritation Response by Visual Observation

While investigating automated image analysis (Section 3.5.1) and reflectance photometry (Section 3.5.5), the capability of the human eye versus the instruments examined was subjectively compared. It was found that for gathering overall skin irritation information (lesion color, height of swelling, and texture), the human eye was as perceptive to slight differences between lesions as either instrument. The problem with visual observations has been in quantifying the differences observed with consistent objectivity. The Draize system is a semiquantitative method that uses the sensitivity of the human eye over a range of irritation responses. Its utility in MREF Protocol 22 has been limited because the lesions produced have typically exhibited irritation at the upper limit of the Draize scale.

As a first-tier screen, MREF Protocol 22 was developed to determine on a binomial (pass/fail) basis the efficacy of a candidate relative to the M258A1 standard decontamination system. If the only conclusion required by the screen is to pass or fail a candidate decontamination system, then a statistic based on a binomial response should be sufficient in evaluating contralateral degrees of irritation.

A binomial response method was developed for visually comparing irritation based on lesion color and height of edema. The comparison was made between contralateral lesions at each time to decontamination. Attempts were made in the comparisons to not let relative lesion area influence the selection of the more irritated skin site. Contralateral lesions exhibiting visually equivalent degrees of irritation were scored as equivalent and were subsequently excluded from analysis.

With the ties thus excluded, an irritation ratio was formed for each time to decontamination by dividing the numerator (the incidence of left-side candidate-decontaminated lesions that exhibited worse irritation than their

contralateral M258A1 decontaminated lesions) by the denominator (the total number of qualifying lesion pairs). The ratios were compared with the theoretical expected fraction of 0.5 for the null hypothesis using analysis of means tests (one-sided) at alpha = 0.05 (Ott, 1975)[7]. Limits of the irritation ratio for accepting the null hypothesis that the candidate decontamination system prevented irritation as well as or better than the M258A1 standard decontamination system were calculated. The limits are presented in the following table as a function of the number of qualifying lesion pairs.

LOWER AND UPPER IRRITATION RATIO AND INCIDENCE LIMITS FOR EQUIVALENCE WITH 0.5 (ALPHA = 0.05)

Number of Lesion Pairs	Ratio		Incidence	
	Lower Limit	Upper Limit	Lower Limit	Upper Limit
72	0.385	0.615	28	44
71	0.384	0.616	28	43
70	0.383	0.617	27	43
69	0.382	0.618	27	42
68	0.381	0.619	26	42
67	0.380	0.620	26	41
66	0.379	0.621	26	40
65	0.378	0.622	25	40
64	0.378	0.623	25	39
63	0.377	0.623	24	39
62	0.376	0.624	24	38
61	0.375	0.625	23	38
60	0.373	0.627	23	37
59	0.372	0.628	22	37
58	0.371	0.629	22	36
57	0.370	0.630	22	35
56	0.369	0.631	21	35
55	0.368	0.632	21	34
54	0.367	0.633	20	34
53	0.365	0.635	20	33
52	0.364	0.636	19	33
51	0.363	0.637	19	32
24	0.300	0.700	8	16
23	0.296	0.704	7	16
22	0.291	0.709	7	15
21	0.286	0.714	7	14
20	0.281	0.719	6	14
19	0.275	0.725	6	13

LOWER AND UPPER IRRITATION RATIO AND INCIDENCE LIMITS FOR EQUIVALENCE WITH 0.5 (ALPHA = 0.05) (Continued)

Number of Lesion Pairs	Ratio		Incidence	
	Lower Limit	Upper Limit	Lower Limit	Upper Limit
18	0.269	0.731	5	13
17	0.262	0.738	5	12
16	0.255	0.745	5	11
15	0.247	0.753	4	11
14	0.238	0.762	4	10
13	0.228	0.772	3	10
12	0.217	0.783	3	9
11	0.205	0.795	3	8
10	0.190	0.810	2	8
9	0.173	0.827	2	7
8	0.154	0.846	2	6
7	0.130	0.870	1	6
6	0.100	0.900	1	5
5	0.062	0.938	1	4
4	0.010	0.990	1	3
3	-0.066	1.066	0	3

The equation used was

$$p_{ij} = \overline{p_i} \pm Z_{\alpha}\sqrt{\overline{p_i}(1-\overline{p_i})/M_i} \quad ,$$

where

p_{ij} = the lower or upper limit of the irritation ratio for equivalence with 0.5,

$\overline{p_i}$ = the theoretical estimate for equal irritation, 0.5,

Z_{α} = the independent variable in the standard normal curve ($Z_{0.05} = 1.96$), and

M_i = the number of qualifying (nontied) lesion pairs.

The incidence limits were calculated as the product of M_i and p_{ij}, rounded to the appropriate integer not exceeding the limit.

The table was generated for two useful ranges of M_i, i.e., from 3 to 24 for comparisons with binomial irritation scores at one time to decontamination, and from 51 to 72 for comparisons with binomial irritation scores

combined over all three times to decontamination. The latter comparison was used to determine whether a candidate decontamination system passed or failed the screen.

The binomial irritation response method was tested on the same 24 animals used in Section 3.5.5 at times to decontamination of HD of 0.75, 1.5, and 3.5 min. The distilled water irritation ratio was 24/24 = 1.0 at each time to decontamination. That is, every dose site decontaminated with distilled water was discernibly more irritated than the contralateral M258A1-decontaminated dose site. These results confirmed the Draize total irritation score results (Table 3.5.3) obtained for that study.

Previous work at the MREF has shown a significant difference between distilled water and M258A1 I and II as HD decontaminants at up to 5 min after dosing. Thus, the results of the binomial irritation response analyses in the validation study presented above were expected. We wanted to better determine whether the binomial irritation response was independent of lesion length or whether analyses of the two responses would always coincide in passing or failing the same candidate decontamination systems. That they would not give the same screen results could only happen under two scenarios. The first is when the lesion lengths on the candidate side are equivalent to or less than those on the standard side, but the binomial irritation score is significantly greater than 0.5. The second scenario is when the lesion lengths on the candidate side are significantly greater than those on the standard side, but the binomial irritation is equivalent to or less than 0.50.

Binomial irritation scores were taken during MREF Protocol 22 screenings of four Rohm & Haas (R&H) candidate decontamination systems in MREF Task 85-12. Each contralateral pair of lesions was evaluated by three technicians. Each candidate system was screened on 3 days with one replicate of eight rabbits per day. The same three technicians were not available for the irritation scoring every day, so that the experimental design was only partially blocked by the observing technician.

Binomial irritation scores are presented for four Rohm & Haas candidate systems in Tables 3.5.4 to 3.5.7. The screen was designed to pass candidate decontamination systems that were as good as or better than the M258A1 I and II standard system. Binomial irritation scores with two plus signs (++)

indicate significantly ($P < 0.05$) lower HD irritation due to candidate decontamination relative to the standard. Scores with one plus sign (+) indicate equivalent HD irritation ($P > 0.05$). Scores with a minus sign (-) indicate significantly higher HD irritation associated with the candidate. Screen results were based on the total irritation scores (by observer) for each candidate system.

An analysis of means among the three observers' total irritation scores showed them to be internally consistent ($P > 0.05$) for each candidate system. That is, based on incomplete blocking of the data, the observers were consistent among themselves in scoring.

A comparison of these binomial irritation results with lesion length analyses is presented in Table 3.5.8. The binomial irritation results in Table 3.5.8 are based on scores made by an observer present on all 3 replicate days of screening. Results of the two methods did not appear to corroborate totally with each other. Both methods passed candidate systems RH1-86, RH4-86, and RH5-86. Candidate system RH6-86 passed the lesion lengths analysis, but failed the binomial irritation response test. Apparently, the lesions were of equivocal length, but the M258A1-decontaminated HD dose sites were less erythemic and/or edematous than the HD dose sites decontaminated with RH6-86. Thus, after examining only four candidate decontamination systems, we detected one case in which the binomial irritation response did not confirm the lesion lengths as a screening parameter. It was concluded that the two responses could be independent and might be used in conjunction as separate indices of the efficacies of candidate systems relative to the standard system in future screening.

In summary, the binomial irritation response method seemed promising as a technique for contralaterally comparing the effectiveness of decontamination systems of HD independently of lesion size. More test comparisons will be needed to assess its full applicability.

3.5.7 Manual Planimetry

As previously discussed in Section 3.5.1, previous experience with an automated image analyzer demonstrated the superiority of the human eye over

digital picture processing in detecting HD lesion boundaries. It was decided to investigate the feasibility of using manual planimetry to measure vesicant lesions on rabbit backs.

A reel digitizer and Sigma-Scan Scientific Measurement Software (Jandel Scientific, Sausalito, CA) was purchased for use with an IBM personal computer. The reel digitizer was a 20 cm by 29 cm by 4 cm plastic box containing two reel-type potentiometers attached by two nylon strings to a drawing pen. The drawing pen had a touch-sensitive tip that required depressing for activation of the digitizer. With the box held stationary, movements of the activated pen were recorded on the computer monitor. The software package could be used to measure the distance between two points, to integrate areas within a closed region, and to determine X/Y coordinates. The instrument was calibrated for distance using a ruler and for area using a 100 cm^2 square drawn on paper.

We initially tried to measure lesion lengths, widths, and areas on live, anesthetized rabbits in a dosing hood. We tested the method with the same 24 animals used in Section 3.5.5 with 0.75, 1.5, and 3.5 min as times to decontamination of HD. The planimeter box was held by a ring stand and was placed in the hood at the approximate level of the rabbit back. Having opened both stanchion sides, we attempted to identify and mark lesion extremities for length and width and to circumscribe lesion boundaries with the drawing pen. This procedure was complicated by the compliance of the skin, i.e., the surface was too soft to allow registration of the lesion extremities by depression of the pen on the skin. Also, attempts to draw the lesion boundaries were unsuccessful due to skin mobility with pen movements. The curvature of the rabbit back required that the rabbit be repositioned for each side of measurements. This was necessary in order to keep the reel strings from contacting skin and generating artifact in pen position. These difficulties made the entire procedure cumbersome and labor intensive.

We tried covering each lesion with a 5-cm glass Petri dish, thereby providing a hard, flat surface on which to draw lesion dimensions. However, use of the dish either flattened the lesions, which rendered them larger in appearance than they actually were, or totally obscured the lesions. Visualizing the lesions was made even more difficult due to working at arm's length

in the hood. We decided that the procedure was not practical when used on the live rabbit restrained in a stanchion in a hood.

We decided to evaluate the feasibility of measuring lesion parameters from projected 35-mm color photographic slides. Photographs were taken of each animal's dorsum. A ruler was positioned in the camera field of view for later calibration. The rabbit was positioned so that its back was at right angles to the camera. The developed slides were projected at an angle onto a mirror so that the dorsum images were reflected onto a flat, white table top. The image size was adjusted at the projector lens so that all regions of the dorsum image could be reached by the drawing pen with the planimeter box kept to the side of the drawing area.

The lesion lengths and widths were measured in millimeters. Data were collected with relative ease, on approximately one rabbit every 3 min. Imaging factors were determined by measuring the distance between the 0 and 50-mm marks on the ruler in each photograph and by dividing that distance by 50 mm. Lesion lengths and widths were corrected by dividing them by the imaging factor determined for that photograph. Each lesion length or width obtained by planimetry on photographs was paired with the value previously obtained by ruler on the live animal per MREF Protocol 22. Statistical contrasts were performed at each dose site for length and width using paired t tests (two-sided) at alpha = 0.05. A significant difference between planimeter and ruler measurements at a given dose site was interpreted as an unacceptable error in the photography/planimetry method at that dose site, since the ruler measurements were regarded as the validated response.

Lesion lengths and widths, corrected by the respective image-sizing factor, are presented in Tables 3.5.9 and 3.5.10 for the 24 animals used. Measurements obtained using a ruler on the same lesions are presented in Tables 3.5.11 and 3.5.12. The same data from both methods are summarized as mean lengths and widths in Table 3.5.13. Results of the paired t tests are also presented in Table 3.5.13, which shows that manual planimetry significantly underestimated the lengths of all M258A1 I and II decontaminated lesions and the nondecontaminated control lesion. The method was accurate on the longest lesions, i.e., at distilled water-decontaminated sites. The method also significantly underestimated all lesion widths except the

smallest, i.e., the site decontaminated at 0.75 min with M258A1 I and II. Based on the above evidence, we decided that the photography/planimetry method was not an adequate tool to substitute for the ruler measurements.

There are several possible reasons why the method underestimated the values obtained by ruler. First and primarily, the photographs did not reveal edema boundaries. Except for trypan blue translocations, there was no visual index of lesion boundaries in the photographs. Since the area of trypan blue was always contained within the area of edema, the lesion size estimates based on trypan blue boundaries were smaller than the ruler measurements, which were based on edema boundaries. Apparently the difference between methods in estimating lesion lengths was least for the longest lesions, i.e., those on which distilled water had been the decontaminant. A second reason for the photography/planimetry method underestimation may have been due to photography at angles other than 90 degrees to the rabbit backs. We had attempted to minimize this effect by supporting the rabbits' abdomen and by orienting the back toward the camera. However, total elimination of the curvature of the back surface was not possible. If back curvature were a significant factor, one would expect it to have the greatest effect on the accuracy of the method in estimating the longest lesions. Since estimates of the longest lesions (>30 mm) were statistically equivalent by the two methods, we concluded that back curvature was not a likely source of error.

In summary, we feel that the use of manual planimetry on live rabbits was not a practical substitute for measurement by ruler. Using the method on projected photographs of rabbit backs also rendered unsatisfactory readings of lesion lengths and widths.

4.0 DISCUSSION

All phases of Optimizations A and D resulted in significant improvements in the base protocol, which is a screen for testing decontamination systems for topical exposure to vesicant chemical surety materiels. Optimization B validated contralateral comparisons and Optimization C validated the use of bulk M258A1 materials in the screen. Results of Optimization E recommended

two techniques for further study, i.e., reflectance photometry and the visually observed binomial irritation response.

4.1 OPTIMIZATION A

Under Optimization A, analyses for HD and L in head space air samples taken over the backs of dosed rabbits demonstrated that animals could be decontaminated and safely removed from dosing hoods at 4 hr after dosing. Studies showed that washing with 0.5 percent NaClO was completely effective in decontaminating/removing any residual HD to a nondetectable level. The procedure was also completely effective in reducing residual L to sub-TWA levels.

Decontamination site skin was not further irritated by 0.5 percent NaClO followed by three rinses with distilled water. The ability of contralateral comparisons of lesion size estimates to detect the efficacy of M258A1 I and II standard decontamination system relative to distilled water was not adversely affected. Instead, the model's sensitivity was significantly improved by performing the NaClO decontamination at 4 hr after dosing for both HD and L. That is, contralateral differences in lesion lengths were increased by an enhanced efficacy of M258A1 I and II when decontamination occurred at 4 hr relative to 24 hr.

Shortening the NaClO decontamination time by 20 hr may affect the outcome of future tests. A candidate decontaminant that tests as equivalent to M258A1 in the current screen may fail the modified screen unless it too demonstrates increased efficacy with the early decontamination process synergistically. However, the adsorbent-type carbonaceous resins that have predominated the candidates in MREF Protocol 22 tests would be more likely to pass the modified screen, since decontaminating with NaClO at 4 hr shortens the time for HD to desorb and enter the skin. This raises the opposite problem, that the modified screen might also pass desorbant candidates that would have failed the unmodified screen. The issue here is the expected length of time in the battlefield between hasty decontamination with the standard personal kit and rendezvous with a chemical squad for deliberate decontamination. If that period is less than 4 hr, then the screen should be

modified so as to enhance detection of short-term superiority in candidates. If deliberate decontamination is expected to be delayed for as much as a day, then the screen should remain as it is in order to avoid unnecessarily stringent comparisons against a standard exhibiting increased efficacy at the shorter decontamination time.

The current safety data must be regarded only as an initial base for justifying removal of animals from the hoods. Periodic redemonstration of sub-TWA levels of vesicant must be performed in order to maintain the safeness of the procedure. It was recommended that an extra group of eight animals be included in the first and every subsequent eighth screening run involving a particular vesicant, and that this group be dosed, decontaminated with 0.5 percent NaClO, rinsed three times with distilled water, and tested for offgassing of HD or arsenicals as described in Optimization A methodology. This frequency of safety checks corresponds to approximately two safety assessments per month of continuous screening and for the first screening run if operations have been discontinued for a time period.

It was therefore recommended that the 4-hr after dosing decontamination with the distilled water rinses be substituted for the 24-hr procedure in the MREF Protocol 22 screen for candidate decontamination systems against vesicants. It was further recommended that animals be removed from dosing and holding hoods following the 4-hr decontamination and placed in conventional animal housing for the remainder of the study. The new procedure not only will provide for cost savings in performing the screen, it will also render a methodology for testing candidate decontamination systems versus both components of the M258A1 standard system against L, which was not possible using the current version of MREF Protocol 22 since the screen for L could not be validated.

4.2 OPTIMIZATION B

Under Optimization B, a multifactor ANOVA demonstrated the expected trend of increased size with time prior to decontamination with the M258A1 I and II system for both HD and L. In the HD study which involved prevalidated times of 1.0, 3.0, and 5.0 min to decontamination, significant anterior-

posterior positional effects were evident in lesion lengths, and significant side effects were evident in lesion widths. These effects were not evident in the HD study using times of 1.25, 5.0, and 10.0 min, and are therefore somewhat suspect. The quadratic position effect in lengths was stronger ($P < 0.01$) than the side effect in widths ($P < 0.05$) and must be considered a more serious factor in the experimental model, especially since lesion length is the primary response used in comparing decontamination system efficacies. Since the validated experimental model for the MREF Protocol 22 screen using HD defines the middle two dose sites to be decontaminated at 3.0 and 5.0 min, lesion length estimates at those times will have predictably greater length components due to position than those at 1.0 min and 24 hr to decontamination.

The important conclusion from Optimization B results is that the MREF Protocol 22 experimental model remains validated as a screen based on contralateral contrasts. Efficacies for candidate and standard decontamination systems against HD or L exposure may be compared using lesion length as the primary response for analysis. There are no statistically significant effects due to the side of the animal at which a dose is applied and decontaminated. Lesion area may be used as a confirmational response that is also independent of side.

Based strictly on data from the HD study using validated times, lesion length was the only measurement significantly ($P < 0.01$) affected by position. Position-dependent lesion length correction terms can be generated by subtracting the overall mean ($n = 256$) lesion length of 21.5 mm from each marginal length mean ($n = 64$) by position (Table 3.2.11) as follows:

CORRECTION TERMS FOR ADJUSTING BY POSITION THE LESION LENGTH MEANS FROM MREF PROTOCOL 22 SCREENS USING HD

Position	Time to Decontamination	Mean Length (mm)	Correction Term (mm)
1	1.0 min	21.0	-0.5
2	3.0 min	22.3	0.8
3	5.0 min	21.9	0.4
4	24 hr	20.6	-0.9

These terms might be subtracted from screen results to more accurately compare lesion length means across times to decontamination. Of

course, including them would not change the results of contralateral comparisons or the conclusions drawn from the analyses. The terms would merely aid in the interpolation of the plotted data.

Similarly, in the L study, significant ($P < 0.001$) effects of position and position interacted with time were evident in lesion widths and areas. Correction terms for widths and areas were calculated by position from Table 3.2.19 for L as follows:

CORRECTION TERMS FOR ADJUSTING BY POSITION THE LESION WIDTH AND AREA MEANS FROM MREF PROTOCOL 22 SCREENS USING L

		Widths		Areas	
Pos.	Time to Decontamination	Mean Width(mm)	Correction Term(mm)	Mean Area(mm^2)	Correction Term(mm^2)
1	30 sec	7.9	0.6	147.0	17.8
2	60 sec	6.7	-0.6	116.1	-13.1
3	120 sec	6.7	-0.6	112.4	-16.8
4	24 hr	7.9	0.6	141.3	12.1

These terms might be subtracted from screen results to more accurately compare lesion width and area means across times to decontamination. Results of the contralateral contrasts would not be affected by these terms.

Whether the position effects change significantly as a function of seasonal changes in rabbit hair growth is not known. Thus, we hesitate to recommend adjustment of the data in MREF Protocol 22 screen results on a routine basis.

4.3 OPTIMIZATION C

Under Optimization C, the preferred use of M258A1 I and II components from bulk packaging was shown to be statistically equivalent to using the M258A1 I and II field kit in decontaminating HD. Thus, we recommend the continued use of M258A1 I and II components from bulk packaging to minimize costs in performing MREF Protocol 22 screens of HD decontamination systems.

A study for comparing bulk versus kit packaging against L was invalidated by a statistically significant ($P < 0.05$) difference between mean

lesion lengths at the identically treated 24-hr control dose sites. In view of the current lower emphasis associated with L, we felt that a repeat of the bulk versus kit study was not warranted. In addition, no statistically significant differences were noted with any of the contralateral comparisons at the validated times used in MREF Protocol 22 (30, 60, and 120 sec) for lesion lengths, which are the measurements used to pass or fail a candidate.

4.4 OPTIMIZATION D

Under Optimization D, the ratio of HD lesion lengths to 24-hr control lengths was shown to be an inappropriate parameter for determining optimal M258A1 I and II decontamination times. The regression value of length ratio with time showed that the minimal length ratio was greater than 0.25; thus, optimal decontamination times (defined at times for length ratios of 0.25, 0.50, and 0.75) could not be determined for a length ratio of 0.25.

Lesion growth ratio, the change in lesion length at a time to decontamination (measured length minus application length) divided by the change in lesion length at 24 hr after dosing, was regressed with time. The times at which the growth ratio was 0.25, 0.50, and 0.75 were approximately 0.5, 2.5, and 5.5 min after dosing, respectively. The regression curve suggested that decontamination with M258A1 I and II could not reduce HD lesion length growth by more than approximately 80 percent of nondecontaminated controls regardless of how quickly it followed dosing.

Based on these values, it is recommended that the validated times to decontamination in MREF Protocol 22 (at 1.0, 3.0, and 5.0 min) be retained.

4.5 OPTIMIZATION E

Under Optimization E, several technologies were examined in an effort to replace the Draize skin irritation score as an index of the efficacy of decontamination systems in reducing HD irritation and to automate the evaluation process while providing a more objective measurement technique. It was found that automated image analysis was not a likely solution due to difficulty in setting a light intensity threshold for normal versus irritated

rabbit skin. Attempts to evenly illuminate the rabbit back failed due to its curvature, which resulted in significant artifacts in image processing. Further investigation in this technology is not recommended at this time.

Thermography was found to be a sensitive tool for detecting thermal differences. However, the lesion thermograms we observed were not consistently shaped. Areas warmer than normal skin commonly excluded the lesion core, presumably due to evaporative cooling. Also, the thermal contrast between normal and irritated skin was not enough to clearly define lesion boundaries. It is felt that thermography does not warrant further investigation unless lesions caused by HD could be used in a second assessment.

A laser Doppler velocimeter appeared to be sensitive to changes in blood flow rate at fixed skin sites as evidenced by a pulsatile output pattern associated with heart beats. The instrument did not seem well suited for this application, however, due to the poor correlation of its output with visually evident erythema. This may have been due in part to variable interference by hair stubble on the rabbit back. Further investigation in this technology is not recommended at this time.

A photopulse plethysmometer was sensitive to changes in local blood volume at specific skin sites. The variability of the instrument's output from site to site, coupled with its sensitivity to operating conditions, masked any evidence of blood volume effects associated with irritation. Further investigation in this technology is not recommended at this time.

The investigation of reflectance photometry was inconclusive. Statistical significance indicated differences in lesion light reflectance for sites decontaminated with distilled water versus M258A1 I and II. Further studies are warranted using this technique under optimal operating conditions and with color filtration.

The binomial irritation scoring response appeared to correlate well with Draize scores in a distilled water versus M258A1 I and II comparison. Analysis of scores from actual screens of decontamination systems suggested that the response was independent of lesion length as an irritation endpoint. It is recommended that this method be adopted as an additional endpoint to lesion lengths in the MREF Protocol 22 screen for vesicant decontaminants, pending its validation using L and additional methodology tests to establish operational parameters and boundaries.

An attempt to use manual planimetry seemed labor intensive and difficult to perform on HD-dosed backs of live rabbits restrained in a hood. Skin compliance and mobility prevented accurate measurement of lesion length and width. Lesion lengths and widths obtained using manual planimetry on photographs of HD-dosed rabbit backs tended to underestimate the measurements obtained by ruler estimates on live animals. A continued effort toward the substitution of photography/planimetry for ruler estimations of HD lesions is not recommended unless used with an edema marker that photographs well. However, it is felt that an investigation of the utility of planimetry to estimate L lesions is warranted since it is the length and width of translocated trypan blue that is measured in MREF Protocol 22 screens of L decontamination systems. Further studies should be undertaken with L in this technology.

5.0 RECORD ARCHIVES

Records pertaining to the conduct of these studies are contained in Battelle Laboratory Record Book Nos. MREF-38, MREF-43, MREF-46, MREF-50, MREF-56, MREF-63, MREF-64, and MREF-76. All pre-study animal quarantine and observation records are on file at MREF. All original data, as well as the original final report, will be maintained at MREF until forwarded to USAMRDC at the conclusion of the project or until microfiched and permanently archived at Battelle.

6.0 ACKNOWLEDGMENTS

The names, role in the study, and highest degree of the principal contributors in this study are presented in the following list:

Name	Title	Degree
Dr. Ronald L. Joiner	Study Director	Ph.D.
Dr. H. Hugh Harroff, Jr.	Chief Veterinarian	D.V.M.
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W. Bruce Keys	Technical Supervisor	M.B.A.
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APPENDIX A

MREF Protocol 23 --- "Optimization of Test and Response Conditions in the Dermal Study for the Assessment and Validation of Decontaminants in Rabbits Against Mustard and Lewisite"

Optimization of Test and Response Conditions in the Dermal Study for the Assessment and Validation of Decontaminants in Rabbits Against Mustard and Lewisite

Study Performed by Battelle Columbus Laboratories,
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3. Sponsor: U. S. Army Medical Research and Development Command

4. Sponsor Monitor: LTC(P) Howard Johnson, USAMRICD

5. Objective:

   To determine optimal test and response conditions in the animal model and experimental methodology for a screen that tests decontaminants against topical exposure to mustard (HD) and Lewisite (L).

6. Optimization Process:

   The animal model and experimental methods described below in Section 7 are used as a base protocol. The optimization scheme investigates several test and response factors in the base protocol, one at a time, and determines conditions that minimize unwanted variability in results and/or that improve cost efficacy. The first optimization step retains or improves conditions in the base protocol, and the optimal version of the protocol is then used as the base in the next optimization step. This succession of protocol versions continues until each test and response condition discussed below in Section 8 is evaluated. The final result is a protocol for screening candidate decontaminants against HD or L that offers maximal sensitivity to differences between candidates and standards with enhanced proficiency.

7. Experimental Design:

   A. Test System

      Albino rabbits were chosen for this study on the basis of the extensive data base available for this species and on the size of the application area for challenges with CSM.

(1) Strain -- New Zealand White (albino) rabbits (male and female), supplied by Kings Wheel Rabbitry.

(2) Initial Weight -- 2.0 to 4.0 kilograms.

(3) Selection -- Animals selected after a minimum 7-day quarantine period are in good physical condition. Rabbits are weighed and randomized into groups based on body weight and sex, having been previously selected for having the least amount of hair growth.

(4) Acclimation -- All animals are held at the Medical Research and Evaluation Facility (MREF) for at least 24 hours prior to study initiation.

(5) Animal Identification -- All animals are ear tattooed to retain positive identification during animal handling and observation. Cage cards are color-coded by sex.

(6) Housing -- Animals are housed individually in stainless steel, slotted cages equipped with automatic watering systems.

(7) Lighting -- Fluorescent lighting, with a light/dark cycle of 12 hours each per day.

(8) Temperature -- Maintained at 70 F (±5 F).

(9) Humidity -- Maintained at 50% (± 10%).

(10) Diet -- Purina Certified Rabbit Chow pellets are available at all times during animal quarantine and holding. No contaminants are known to be present in the feed that would interfere with the results of the study.

(11) Water Supply -- Water is supplied from the public water system and given ad libitum during quarantine and holding. No contaminants are known to be present in the water that would affect the results of the study.

(12) Animal Care During Test -- All animals are housed in stanchions during the treatment period and in individual cages for the remainder of the test period. No food is provided during the treatment period. Water is provided ad libitum.

(13) Laboratory Animal Welfare Practices -- Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture as a Research Facility (Number 31-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHEW Publication No. (NIH) 78-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, Pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544 and P.L. 91-579).

(14) Accreditation -- On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Groups

(1) Size -- Routine screening tests are performed with groups of 8 animals. Group matching is based on individual and total group weight and sex.

(2) Number -- Two groups of animals are used for each series of exposures. One test group receives HD and the appropriate decontamination solutions and the second group receives L and the decontamination solutions.

(3) Design -- Each animal in each group receives a series of 0.5 µl doses of HD or L along the dorsum of the back in the following pattern:

anterior ⟵⟶ posterior

midline	b	x	y	z	b_1	b
		1	2	3	a	

where a = CSM control without decontamination,
b = control for standard decontamination material,
b1 = control for test decontamination material,
x = CSM plus standard decontamination material after minimum time period,
y = CSM plus standard decontamination material after middle time period,
z = CSM plus standard decontamination material after maximum time period,
1 = CSM plus test decontamination material after minimum time period,
2 = CSM plus test decontamination material after middle time period,
3 = CSM plus test decontamination material after maximum time period.

(4) Dose -- The volume of CSM applied at each position is 0.5 µl.

C. Test Material

The M258A1 skin decontamination kit is used as the standard decontamination system against HD and L exposure. Specific test decontamination materials are provided for comparison of effects.

(1) The M258A1 skin decontamination kits are supplied by the USAMRDC/ICD. Bulk components of the M258A1 kits are purchased from Chemtronics Corporation, Swannanoa, NC.

(2) Lewisite and HD are supplied by the USAMRDC/ICD. Purity, appropriate identification (bath number, lot number, state), and stability date are supplied by the USAMRDC/ICD. Purity and stability are confirmed periodically by Battelle for material stored at Battelle.

(3) Surety, security, and safety procedures for the use of L and HD are thoroughly outlined in facility plans, in personnel requirements for qualifications to work with CSM, and in CSM storage and use standard operating procedures.

D. Preparation of Animals

(1) Hair Clipping -- All animals are acclimated in approved cages at the MREF for at least one day before use. Study animals are closely clipped from withers to rump with care to avoid skin damage. An Oster Model A-2 animal clipper with a No. 40 blade, or equivalent, is used to clip animals approximately 24 hours prior to the intended use. Animals are reclipped, if necessary, after anesthesia has been induced to prevent shielding of exposure sites by hair stubble.

(2) Anesthesia -- Rabbits are anesthetized prior to treatment by the intermuscular administration of a mixture of Ketamine and Rompun (17.5 mg/kg of Ketamine and 5.0 mg/kg of Rompun). The unconscious rabbits are placed in prone position in metal stanchions. Animals are then placed inside exposure hoods and the hood sash positioned to maintain proper air flow past the rabbit noses.

E. Application of Vesicants

(1) CSM (0.5 µl of L or HD) is applied to each of the designated spots on the back of each rabbit as a small streak (approximately 1 cm in length) with a microliter syringe. Lewisite is delivered using a Hamilton microliter syringe equipped with a special platinum barrel and a tungsten plunger. Standard stainless microliter syringes are used to apply HD.

(2) Agent and decontaminates are applied as described in 7.B(3).

F. Application of Decontaminants

(1) Candidate decontaminants are compared directly with standard decontaminants on each animal. All candidate decontaminants are compared with the components of the dual-component M258A1 skin decontamination kit currently fielded by the U. S. Army.

(2) The duration of exposure before beginning decontamination is a critical factor.

(a) Lewisite decontamination with standard and experimental materials begins at the respective dosing sites at 30, 60, and 120 seconds after CSM application.

(b) Mustard decontamination begins at the dosing sites at 1.25, 5, and 10 minutes after CSM application.

(c) Modifications to the time sequences may be made to provide more distinctly graded readings, as necessary.

(3) The mechanical method used to remove the applied CSM is another critical factor. Each decontaminant is applied according to the manufacturer's "field use" directions or with the use of a pad or cloth fastened to a tongue depressor as described below.

(a) The standard M258A1 skin decontamination kit uses two pre-packaged components for decontamination. The contents of packet I are applied prior to that of packet II as directed by the instructions on each packet.

(b) An alternative to using the pre-packaged material is to prepare towelettes with the appropriate packet I or II solutions from bulk materials. The cloth used to make

Component I is cut into appropriate lengths (based on the surface area ratio between the exposed area on the rabbit back and the exposed area of a soldier) and attached to a tongue depressor. The assembly is then wetted from a syringe containing the liquid portion of Component I recently drawn up from a freshly opened container. The amount of liquid for Component I is proportional to the amount of cloth used as detailed in the production MIL specifications for the M258A1 kit. Preparation of the Component II decontaminant is similar but differs in that the Component II cloth is used (which has been impregnated with chloramine B) for receiving the liquid portion of Component II.

(c) Candidate decontaminants are administered according to the manufacturer's "field-use" directions. If directions are not available, the application procedure will be determined for each candidate by the USAMRDC COTR and the MREF Manager.

(d) A standardized application system for candidate decontaminants without manufacturer directions could use an applicator of the Component I-type with a predetermined amount of liquid applied to the applicator (determined for each candidate by the USAMRDC COTR and the MREF Manager).

(4) The sequence and timing of application of the decontaminants are based on the instructions for field application of the M258A1 kit materials or of the candidate decontaminants.

(a) For the M258A1 kit, an applicator pad wetted with M258A1 Component I is wiped briskly within the exposure area for 10 seconds in a back and forth motion perpendicular to the spine. The Component II wetted pad is applied 65 seconds later in a similar manner for 10 seconds.

(b) If not specified by the manufacturer, candidate liquid decontaminants are applied in a manner similar to that for the M258A1 materials. The applicator or prepared wetted pad is wiped briskly but not harshly for 10 seconds within the exposure area in a back and forth motion perpendicular to the spine. A second application 65 seconds later may be applied as specified by the USAMRDC COTR and MREF Manager.

(c) Immediately after use, each applicator is placed into a container of 5% sodium hypochlorite.

(5) Experimental and standard decontamination sites are washed at either 4 or 24 hours post-exposure with 5% sodium hypochlorite followed by 3 washings with distilled water. The time of the initial sodium hypochlorite washing may be changed as necessary.

(6) Animals are removed from the hood after decontamination and placed in standard rabbit caging for the remainder of the experimental period.

G. Lesion Evaluation

(1) Dye Injection -- After decontamination at 20-24 hours post-exposure, each animal is given a 1-ml intramuscular injection (in each thigh) of a 3% suspension of Trypan blue dye in saline. The dye requires at least two hours to translocate throughout the damaged vessels of the exposed areas. The dye forms a dark blue marking of the lesion against the contrasting pale blue of adjacent normal skin. A pink halo may extend for 2-4 mm wider than the blue zone, which presumably is indicative of active hyperemia.

(2) Anesthesia -- Approximately 2-4 hours after administration of the dye, the test animals are anesthetized by a dose of Ketamine/Rompun (17.5 mg/kg of Ketamine and 5.0 mg/kg of Rompun).

(3) Lesion Size Determination -- After anesthesia (at approximately hour 24 to hour 28), the lesion at each exposure site is visually compared with the contralateral lesion at the corresponding time interval for the standard M258A1 decontamination kit components. The observer estimates the length and width of each affected area by matching the two axes with a series of references with known lengths and areas. Representative lesions are recorded photographically.

(4) Euthanasia -- After lesion evaluation, the test animals are killed by administering T-61.

H. Disposal of Experimental Animals

(1) Packaging for Disposal -- The euthanitized animals are decontaminated with 5% sodium hypochlorite, placed into double plastic bags that are sealed, and the bags are removed from the hood to await sampling for proof of decontamination analysis.

(2) Disposal -- All packaged animals are incinerated after proof of decontamination.

I. Necropsy and Histopathology

No tissue samples are to be saved, and all animals carcasses are to be decontaminated and discarded.

8. Optimizations:

A. Removal of Animals from Hoods on Day of Dosing

(1) Two consequences of removing the animals from hoods 4 hours after dosing are examined:

(a) The hazards of personnel exposure to vesicant evaporated from animal backs either after or without decontamination with 5% sodium hypochlorite and rinsing with water

(b) The effect on local irritation and lesion size by decontamination with 5% sodium hypochlorite and rinsing

with water at 4 hours post-dosing, followed by a 20-hour period prior to lesion size estimations.

(2) Two series of two groups each (8 rabbits per group) are used. The groups are defined below:

	Group	N	Vesicant	Std Decon Used on Right Side	Time to Sodium Hypochlorite Decon (hrs Post-Dose)
Series I					
	1	8	HD	M258A1 I, II	24
	2	8	HD	M258A1 I, II	4
Series II					
	3	8	L	M258A1 I, II	24
	4	8	L	M258A1 I, II	4

(3) Animals are dosed at 7 sites per base protocol. Right side dose sites are decontaminated with the standard per base protocol, and left side sites are not decontaminated. Four hours after dosing, animals in Groups 2 and 4 are decontaminated at every dose site twice with 5% sodium hypochlorite and rinsed three times with distilled water. Animals in Groups 1 and 3 are not decontaminated or rinsed.

(4) Then all animals, while remaining in stanchions in the hood, are placed into plastic bags in pairs (the head of each animal remains outside of the bag). The bags are perforated with pasteur pipettes to supply replacement head space air withdrawn through impingers or adsorbent tubes for standard sampling and quantitative analysis for vesicant. Head space air is sampled for one to three hours. The animals are removed from the bags but remain in stanchions in the hood until 24 hours post-dosing. Water is supplied ad libitum. Lesion size estimates are observed and recorded.

(5) Results of head space samples are compared with 10-day TWA's established for each vesicant. If any one or more of the 10 bag sample results per series is greater than the 10-day TWA, then that series is repeated. Any procedure for which all of 10 bag sample results for each replicate are less than the 10-day TWA is carried forward as an optimization to the base protocol.

(6) Lesion size estimates are made at all dose sites. The experimental design analysis is unpaired t-tests between groups per series. The responses examined are the size and irritation

differences between left and right lesions at the same anterior/posterior position in each series' group. The lack of a statistically significant (i.e., if P>0.05, one-tailed) change in the difference between contralateral lesions at any of dosing positions 1, 2, or 3 retains the early decontamination and rinse at 4 hours post-dosing for that vesicant/standard decontaminant combination in the base protocol, provided that safety considerations outlined above are met.

B. Positional Effects in Test Design

(1) The effect of the position of the dose on the rabbit back is examined using a 3-way factorial experimental design. The factors to be assessed are side (left/right) and anterior-posterior position (1, 2, 3, 4) at every time to decontamination (1.25, 5.0, 10.0 minutes, and 24 hours for HD; 30, 60, 120 seconds, and 24 hours for L).

(2) Each animal is dosed and decontaminated the same on both sides at each position. Times to decontamination are varied with position from group to group. Four groups of 8 rabbits per group with 8 test sites per rabbit equals 256 test sites in the design. Group definitions are as follows:

			Position			
Group	N	Side	1	2	3	4
1	8	Both	s	m	L	24 hr
2	8	Both	m	L	24 hr	s
3	8	Both	L	24 hr	s	m
4	8	Both	24 hr	s	m	L

where s = shortest, m = middle, l = longest exposure periods, and 24 hr = 24 hours exposure period prior to decontamination.

All other experimental details, including dosing and decontamination techniques and lesion size estimations, are the same as previously described. Lesion size estimate is the response for analysis. Irritation is not an endpoint for evaluation, and lesions are not recorded photographically.

The absence of statistically significant effects (i.e., if P>0.05, one-tailed) due to position and side at each time to decontamination retains the animal dosing scheme in the base protocol. If significant positional effects are detected at any time to decontamination, then a joint decision by the MREF

Manager and the USAMRDC COTR is made on whether and how to modify the dosing scheme in view of the magnitude of the effects.

C. M258A1 Standard Kit Materials Packaging

(1) The effect of standard M258A1 decontamination materials packaging is examined. M258A1 components supplied in bulk form are compared contralaterally to the M258A1 field kit components supplied in individual packaging and freshly opened prior to application.

(2) Contralateral comparisons are made at one anterior-posterior position on the rabbit back. Both sides receive standard decontamination with M258A1 at the shortest exposure period in the base protocol. Non-decontaminated control sites are included at a second position on each animal to insure contralateral uniformity in test skin.

(3) Bulk materials are prepared and applied to the dose site as described in Section 7.F. above. The field kits are prepared per kit instructions, except that an amount of decontamination pad and materials equivalent to the bulk preparation are cut away and fastened to a tongue depressor. All other details in the base protocol remain intact, including decontamination wiping action, time of wiping, etc.

M258A1 Decontamination

Side	Packaging	Yes	No
Right	Kit		
Left	Bulk		

Anterior ⟷ Posterior

(4) The experimental design analysis is a paired t-test between lesion size estimates from the two packaging forms of standard decontamination materials. The test is invalidated if the non-decontaminated lesion size means are statistically significantly different. The group size is 20 rabbits per vesicant.

The absence of a statistically significant difference (i.e., if P>0.05, one-tailed) between lesion sizes using different packaging forms, or an increase in lesion size using bulk materials relative to using kits, retains the use of bulk materials in the base protocol. If lesions from using kit material are significantly smaller than those from using bulk material, then a joint decision by the MREF Manager and the USAMRDC COTR is made on which packaging form to adopt in view of the magnitude of the difference.

D. Factorial Design to Optimize Standard Decontamination

Three factors - time to decontamination following exposure, quantity of standard decontaminant used, and duration of wiping with decontaminant - can be varied in a factorial design to determine the effects of each factor and their interactions on lesion size estimates. The full analysis is not necessary for the M258A1 kit, since the amount of decontaminant and the duration of wiping are fixed by kit instructions. Also, determining for L a set of times to effective decontamination is not practical since the present shortest period, 30 seconds, is already too long a delay to effectively begin decontamination. Thus, the only factor requiring analysis for the M258A1 kit under this protocol is the time to decontamination parameter for HD.

(1) For HD decontaminated with M258A1, the periods between dosing and initiation of decontamination are modified in sets of three while holding the other factors fixed per the base protocol.

(2) The entire rabbit back with 6 dosing sites is used. The right side is dosed at 3 sites, and each is decontaminated with M258A1 kit components 24 hours later. The left side is dosed at 3 contralateral sites and identically decontaminated at 3 test periods to be prescribed.

(3) Groups of 8 rabbits are used per set of test periods. Shortest, middle, and longest test periods are modified until lesion size estimates are obtained approximating 25%, 50%, and 75%, respectively, of each contralateral 24-hour exposure lesion size.

E. Improved Draize Scoring

Methods for indexing erythema and/or edema due to topical vesicant insult are evaluated. The methods to be tested include but are not limited to infrared photometry of whole lesions.

9. Necropsy and Histopathology:

No tissue samples are to be saved, and all animals carcasses are to be decontaminated and discarded.

10. Records to be Maintained:

A. CSM accountability log and inventory

B. Dose preparation and administration

C. Animal data

D. Experimental parameters and test conditions

E. Lesion observations and evaluations

F. Results of decontamination monitoring

G. Confirmation of disposal

11. Statistical Methods:

The evaluation of the relative effectiveness of the timed sequence of test decontamination solutions or powders is done by comparing those results with the corresponding standard M258A1 controls. At the same time intervals, the corresponding lesion intensity, as estimated by the length, width, and/or area of lesion involvement, for the test decontamination solution or powder plus CSM is compared to the lesion intensity from the M258A1 decontamination kit components.

Significant differences in the intensities or areas of involvement measured in the one-sided, paired t-test can be used to classify a test decontamination material as "less effective" or "equal to or more effective" than the M258A1 standard. If the test material is as effective as the standard, it may warrant another series of tests at different application intervals after exposure, with different decontaminating time sequences, or with different amounts of test decontamination materials.

12. Reports:

A draft final report is prepared and submitted for review by the USAMRDC COTR within 30 working days after completion of the task. It includes at least the following:

1. Signature page for key study individuals and their responsibilities
2. Experimental design
3. Animal supplier
4. Test animal selection criteria
5. Test material description and preparation
6. Application procedures
7. Clinical observations
8. Tabulation of response data
9. Statistical methodology
10. Discussion
11. Photographs

A final report that addresses the review comments of the USAMRDC is prepared and submitted within 30 days of receipt of comments.

13. Approval Signatures:

Ronald A Joiner — Study Director — 14 MAY 1985 — Date

H. Hugh Haverkoff, Jr DVM — Chief Veterinarian — 14 May 1985 — Date

Howard C Johnson — USAMRDC COTR — 14 MAY 85 — Date

14. Amendment A - June 12, 1985:

This is to document changes in Protocol 23 (Optimization of Test and Response Conditions in the Dermal Study for the Assessment and Validation of Decontaminants in Rabbits Against Mustard and Lewisite).

1. Page 9, Section 8. Optimization A. (3):

   The concentration of sodium hypochlorite for decontamination of dose sites is changed to 0.5%. This is the highest concentration that does not interfere with the estimation of lesions by exacerbating irritation.

2. Page 9, Section 8. Optimization A. (4) is replaced with the following:

   Then all animals, while remaining in stanchions in the hood, are placed singly into cardboard boxes with the lids removed. Large funnels, approximately 6 inches in largest diameter, are trimmed on opposite sides at their wide ends to fit the curvature of the rabbit back. The funnels are connected at the narrow ends through tygon tubing to impingers or absorbent tubes for standard sampling and quantitative analysis for vesicant. A funnel is positioned over and taped with duct tape onto each rabbit back. Head space air is sampled for one hour. The funnels are detached, and the animals are removed from the boxes but remain in stanchions in the hood until 24 hours post-dosing. Water is supplied ad libitum. Lesion size estimates are observed and recorded.

3. Page 9, Section 8. Optimization A. (5) is replaced with the following:

   Results of head space samples are compared with 10-day TWA's established for each vesicant. If results reveal no detectable vesicant (limit = 0.2 TWA for HD) from any animal decontaminated at 4 hours post-dosing, then the optimization step will be considered for implementation.

15. Approval Signatures for Amendment A:

Ronald L. Joiner, Ph.D.
Study Director

21 June 1985
Date

H. Hugh Harroff, Jr., D.V.M.
Chief Veterinarian

21 June 1985
Date

LTC (P) Howard C. Johnson, D.V.M.
Sponsor Monitor

21 June 85
Date

APPENDIX B

MREF Protocol 22 --- "Assessment of Liquid or Powder Decontaminants in Rabbits Against Dermal Applications of Mustard and Lewisite"

Assessment of Liquid or Powder Decontaminants in Rabbits Against Dermal Applications of Mustard and Lewisite

Study Performed by Battelle Columbus Laboratories,
505 King Avenue, Columbus, Ohio 43201

1. Study Director: Ronald L. Joiner, Ph.D.

2. Veterinarian: H. Hugh Harroff, Jr., D.V.M.

3. Sponsor: U.S. Army Medical Research and Development Command

4. Sponsor Monitor: LTC(P) Howard Johnson, USAMRICD

5. Objective:

   To develop a quantitative animal model and experimental method for screening and testing liquid or powder experimental decontaminants against the standard dual-component M258A1 skin decontamination kit or Fuller's Earth following mustard (HD) or Lewisite (L) exposure.

6. Experimental Design:

   A. Test System

      Albino rabbits were chosen for this study on the basis of the extensive data base available for this species and on the size of the application area for multiple challenges with CSM.

      (1) Strain -- New Zealand White (albino) rabbits (male and female), supplied by Kings Wheel Rabbitry.

      (2) Initial Weight -- 2.0 to 4.0 kilograms.

      (3) Selection -- Animals selected after a minimum 7-day quarantine period are in good physical condition. Rabbits are weighed and randomized into groups based on body weight and sex, having been previously selected for having the least amount of hair growth.

      (4) Acclimation -- All animals are held at the Medical Research and Evaluation Facility (MREF) for at least 24 hours prior to study initiation.

(5) Animal Identification -- All animals are ear tattooed to retain positive identification during animal handling and observation. Cage cards are color-coded by sex.

(6) Housing -- Animals are housed individually in stainless steel, slotted cages equipped with automatic watering systems.

(7) Lighting -- Fluorescent lighting, with a light/dark cycle of 12 hours each per day.

(8) Temperature -- Maintained at 70F (±5F).

(9) Humidity -- Maintained at 50% (± 10%).

(10) Diet -- Purina Certified Rabbit Chow pellets are available at all times during animal quarantine and holding. No contaminants are known to be present in the feed that would interfere with the results of the study.

(11) Water Supply -- Water is supplied from the public water system and given ad libitum during quarantine and holding. No contaminants are known to be present in the water that would affect the results of the study.

(12) Animal Care During Test -- All animals are housed in stanchions during the treatment period and in individual cages for the remainder of the test period. No food is provided during the treatment period. Water is provided ad libitum.

(13) Laboratory Animal Welfare Practices -- Battelle's Animal Resources Facilities have been registered with the U.S. Department of Agriculture as a Research Facility (Number 31-21) since August 14, 1967, and are periodically inspected in accordance with the provisions of the Federal Animal Welfare Act. In addition, animals for use in research are obtained only from laboratory animal suppliers duly licensed by the USDA. Battelle's statement of assurance regarding the Department of Health and Human Services policy on humane care of laboratory animals was accepted by the Office of Protection from Research Risks, National Institutes of Health on August 27, 1973. Animals at Battelle are cared for in accordance with the guidelines set forth in the "Guide for the Care and Use of Laboratory Animals" (DHEW Publication No. (NIH) 78-23), and/or in the regulations and standards as promulgated by the Agricultural Research Service, USDA, Pursuant to the Laboratory Animal Welfare Act of August 24, 1966 as amended (P.L. 89-544 and P.L. 91-579).

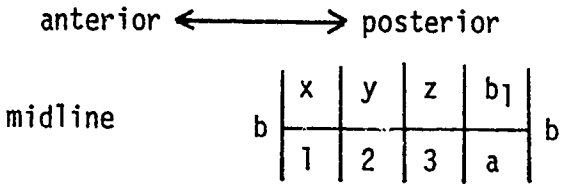
(14) Accreditation -- On January 31, 1978, Battelle's Columbus Division received full accreditation of its animal-care program and facilities from the American Association for Accreditation of Laboratory Animal Care (AAALAC). Battelle's full accreditation status has been renewed after every inspection since the original accreditation. The MREF is a part of the facilities granted full accreditation.

B. Test Groups

(1) Size -- Routine screening tests are performed with groups of 8 animals. Group matching is based on individual and total group weight and sex.

(2) Number -- Two groups of animals are used for each series of exposures. One test group receives HD and the appropriate decontamination solutions or powders and the second group receives L and the decontamination solutions or powders.

(3) Design -- Each animal in each group receives a series of 0.5 µl doses of HD or L along the dorsum of the back in the following pattern:



where a = CSM control without decontamination,
b = control for standard decontamination material,
b1 = control for test decontamination material,
x = CSM plus standard decontamination material after minimum time period,
y = CSM plus standard decontamination material after middle time period,
z = CSM plus standard decontamination material after maximum time period,
1 = CSM plus test decontamination material after minimum time period,
2 = CSM plus test decontamination material after middle time period,
3 = CSM plus test decontamination material after maximum time period.

(4) Dose -- The volume of CSM applied at each position is 0.5 µl.

C. Test Material

The M258A1 skin decontamination kit or Fuller's Earth powder is used as the standard decontamination system against HD and L exposure. Specific test decontamination materials are provided for comparison of effects.

(1) The M258A1 skin decontamination kits are supplied by the USAMRDC/ICD. Bulk components of the M258A1 kits are purchased from Chemtronics Corporation, Swannanona, NC. Fuller's Earth is available commercially from Sigma Chemical Company, St. Louis, MO.

(2) Lewisite and HD are supplied by the USAMRDC/ICD. Purity, appropriate identification (bath number, lot number, state), and stability date are supplied by the USAMRDC/ICD. Purity and stability are confirmed periodically by Battelle for material stored at Battelle.

(3) Surety, security, and safety procedures for the use of L and HD are thoroughly outlined in facility plans, in personnel requirements for qualifications to work with CSM, and in CSM storage and use standard operating procedures.

D. Preparation of Animals

(1) Hair Clipping -- All animals are acclimated in approved cages at the MREF for at least one day before use. Study animals are closely clipped from withers to rump with care to avoid skin damage. An Oster Model A-2 animal clipper with a No. 40 blade, or equivalent, is used to clip animals approximately 24 hours prior to the intended use. Animals are reclipped, if necessary, after anesthesia has been induced to prevent shielding of exposure sites by hair stubble.

(2) Anesthesia -- Rabbits are anesthetized prior to treatment by the intermuscular administration of a mixture of Ketamine and Rompun. The unconscious rabbits are placed in prone position in metal stanchions. Animals are then placed inside exposure hoods and the hood sash positioned to maintain proper air flow past the rabbit noses.

E. Application of Vesicants

(1) CSM (0.5 µl of L or HD) is applied to each of the designated spots on the back of each rabbit as a small streak (approximately 1 cm in length) with a microliter syringe. Lewisite is delivered using a Hamilton microliter syringe equipped with a special platinum barrel and a tungsten plunger. Standard stainless microliter syringes are used to apply HD.

(2) Agent and decontaminates are applied as described in 6.B(3).

F. Application of Liquid Decontaminants

(1) Candidate decontaminants are compared directly with standard decontaminants on each animal. Liquid candidate decontaminants are compared with the components of the dual-component M258A1 skin decontamination kit currently fielded by the U. S. Army.

(2) The duration of exposure before beginning decontamination is a critical factor.

(a) Lewisite decontamination with standard and experimental materials begins at the respective dosing sites at 30, 60, and 120 seconds after CSM application.

(b) Mustard decontamination begins at the dosing sites at 1.25, 5, and 10 minutes after CSM application.

(c) Modifications to the time sequences may be made to provide more distinctly graded readings, as necessary.

(3) The mechanical method used to remove the applied CSM is another critical factor. Each liquid decontaminant is applied according to the manufacturer's "field use" directions or with the use of a pad or cloth fastened to a tongue depressor.

(a) The standard M258A1 skin decontamination kit uses two pre-packaged components for decontamination. The contents of packet I are applied prior to that of packet II as directed by the instructions on each packet.

(b) An alternative to using the pre-packaged material is to prepare towelettes with the appropriate packet I or II solutions from bulk materials. The cloth used to make Component I is cut into appropriate lengths (based on the surface area ratio between the exposed area on the rabbit

back and the exposed area of a soldier) and attached to a tongue depressor. The assembly is then wetted from a syringe containing the liquid portion of Component I recently drawn up from a freshly opened container. The amount of liquid for Component I is proportional to the amount of cloth used as detailed in the production MIL specifications for the M258A1 kit. Preparation of the Component II decontaminant is similar but differs in that the Component II cloth is used (which has been impregnated with chloramine B) for receiving the liquid portion of Component II.

(c) Candidate liquid decontaminants are administered according to the manufacturer's "field-use" directions. If directions are not available, the application procedure will be determined for each candidate by the USAMRDC COTR and the MREF Manager.

(d) A standardized application system for candidate liquid decontaminants without manufacturer directions could use an applicator of the Component I-type with a predetermined amount of liquid applied to the applicator (determined for each candidate by the USAMRDC COTR and the MREF Manager).

(4) The sequence and timing of application of the decontaminants are based on the instructions for field application of the M258A1 kit materials or of the candidate decontaminants.

(a) For the M258A1 kit, an applicator pad wetted with M258A1 Component I is wiped briskly within the exposure area for 10 seconds in a back and forth motion perpendicular to the spine. The Component II wetted pad is applied 65 seconds later in a similar manner for 10 seconds.

(b) If not specified by the manufacturer, candidate liquid decontaminants are applied in a manner similar to that for the M258A1 materials. The applicator or prepared wetted pad is wiped briskly but not harshly for 10 seconds within the exposure area in a back and forth motion perpendicular to the spine. A second application 65 seconds later may be made as specified by the USAMRDC COTR and the MREF Manager.

(c) Immediately after use, each applicator is placed into a container of 5% sodium hypochlorite.

(5) Experimental and standard decontamination sites are washed at either 4 or 24 hours post-exposure with 5% sodium hypochlorite followed by 3 washings with distilled water. The time of the initial sodium hypochlorite washing may be changed as necessary.

(6) Animals are removed from the hood after decontamination and placed in standard rabbit caging for the remainder of the experimental period.

G. Application of Powder Decontaminants

(1) The standard decontamination system for comparison against candidate powder decontaminants is the M258A1 dual-component skin decontamination kit. On specified occasions, a candidate powder decontaminant may be compared to Fuller's Earth. All solid or powder experimental decontamination systems are applied as follows whether being compared to M258A1 or Fuller's Earth.

(2) The standard Fuller's Earth and experimental decontaminants are applied directly to the exposed area.

(3) A pre-weighed quantity of Fuller's Earth (100-150 mg) is placed into capped vials and stored until use.

(4) The Fuller's Earth is applied to the dosed site and rubbed thoroughly over the entire dosing area for 10 seconds with a cotton-tipped swab.

(5) A piece of cardboard is held at one border of the immediate site of powder application to minimize contamination of adjacent dosing sites through air entrapment of excess powder.

(6) Immediately after use, the cotton-tipped swab is placed into a container of 5% sodium hypochlorite.

(7) Candidate powder decontaminants are administered according to the manufacturer's "field-use" directions. If directions are not available, the application procedure will be determined for each candidate by the USAMRDC COTR and the MREF Manager.

(8) Experimental and standard decontamination sites are washed at either 4 or 24 hours post-exposure with 5% sodium hypochlorite followed by 3 washings with distilled water. The time of initial sodium hypochlorite washing may be changed as necessary.

(9) Animals may be removed from the hood after decontamination and placed in standard cages for the remainder of the experimental period.

H. Lesion Evaluation

(1) Dye Injection -- After decontamination at 20-24 hours post-exposure, each animal is given a 1-ml intramuscular injection (in each thigh) of a 3% suspension of Trypan blue dye in saline. The dye requires at least two hours to translocate throughout the damaged vessels of the exposed areas. The dye forms a dark blue marking of the lesion against the contrasting pale blue of adjacent normal skin. A pink halo may extend for 2-3 mm wider than the blue zone, which presumably is indicative of active hyperemia.

(2) Anesthesia -- Approximately 2-4 hours after administration of the dye, the test animals are anesthetized by a dose of Ketamine/Rompun (17.5 mg/kg of Ketamine and 5.0 mg/kg of Rompun).

(3) Lesion Size Determination -- After anesthesia (at approximately hour 24 to hour 28), the lesion at each exposure site is visually compared with the contralateral lesion at the corresponding time interval for the standard M258A1 decontamination kit components or Fuller's Earth. The observer estimates the length and width of each affected area by matching the two axes with a series of references with known lengths and areas. Representative lesions are recorded photographically.

(4) Euthanasia -- After lesion evaluation, the test animals are killed by administering T-61.

H. Disposal of Experimental Animals

(1) Packaging for Disposal -- The euthanitized animals are decontaminated with 5% sodium hypochlorite, placed into double plastic bags that are sealed, and the bags are removed from the hood to await sampling for proof of decontamination analysis.

(2) Disposal -- All packaged animals are incinerated after proof of decontamination.

7. Necropsy and Histopathology:

No tissue samples are to be saved, and all animals carcasses are to be decontaminated and discarded.

8. Records to be Maintained:

   A. CSM accountability log and inventory

   B. Preparation of reagents and dosage administration

   C. Animal data

   D. Experimental parameters and test conditions

   E. Lesion observations and evaluations

   F. Results of decontamination monitoring

   G. Confirmation of disposal

9. Statistical Methods:

   The evaluation of the relative effectiveness of the timed sequence of test decontamination solutions or powders is done by comparing those results with the corresponding standard M258A1 or Fuller's Earth controls. At the same time intervals, the corresponding lesion intensity, as estimated by the length, width, and/or area of lesion involvement, for the test decontamination solution or powder plus CSM is compared to the lesion intensity from the M258A1 decontamination kit components (or Fuller's Earth on special occasion) plus CSM.

   Significant differences in the intensities or areas of involvement measured in the one-sided, paired t-test can be used to classify a test decontamination material as "less effective" or "equal to or more effective" than the M258A1 or Fuller's Earth standard. If the test material is as effective as the standard, it may warrant another series of tests at different application intervals after exposure, with different decontaminating time sequences, or with different amounts of test decontamination materials.

10. Reports:

    A draft final report is prepared and submitted for review by the USAMRDC COTR within 30 working days after completion of the task. It includes at least the following:

    1. Signature page for key study individuals and their responsibilities
    2. Experimental design
    3. Animal supplier

4. Test animal selection criteria
5. Test material description and preparation
6. Application procedures
7. Clinical observations
8. Tabulation of response data
9. Statistical methodology
10. Discussion
11. Photographs

A final report that addresses the review comments of the USAMRDC is prepared and submitted within 30 days of receipt of comments.

11. Approval Signatures:

Ronald L. Joiner, Ph.D.
Study Director

14 May 1985
Date

H. Hugh Harroff, Jr., D.V.M.
Chief Veterinarian

14 May 85
Date

LTC(P) Howard C. Johnson, D.V.M.
USAMRDC Monitor

14 MAY 85
Date

12. Amendment A - January 10, 1986:

This is to document changes in Protocol 22 (Assessment of Liquid or Powder Decontaminants in Rabbits Against Dermal Applications of Mustard and Lewisite).

Page 7, Section 6.F.(5) is replaced with the following:

Experimental and standard decontamination sites are washed at either 4 hours post-exposure with 0.5% sodium hypochlorite solution or at 24 hours post-exposure with 5% sodium hypochlorite solution or both, followed by a minimum of 3 washings with distilled water. The distilled water wastes are performed in order to clean the dose site of decontaminants for lesion observations. The number of washes used is consistent within a study and is recorded.

13. Approval Signatures for Amendment B:

Ronald L. Joiner, Ph.D.
Study Director

14 January 86
Date

H. Hugh Harroff, D.V.M.
Chief Veterinarian

14 January 1986
Date

LTC(P) Howard C. Johnson, D.V.M.
USAMRDC/ICD Monitor

31 Jan 86
Date

14. Amendment B - April 10, 1986:

This is to document changes in Protocol 22 (Assessment of Liquid or Powder Decontaminants in Rabbits Against Dermal Applications of Mustard and Lewisite).

Page 3, Section 6.B.(3)

The pattern for dosing each animal is redefined by the following:

anterior ⟷ posterior

midline

b	x	y	z	a	b
	1	2	3	b_1	

The switching of dose sites "a" and "b_1" was made so that site "a" (CSM control without decontamination) would be on the same side as the standard decontamination control dose sites "x", "y", and "z".

Page 4, Section 6.C.(1) is amended with the following:

Bulk M258A1 components used after March 1986 are purchased from Mine Safety Appliances, Murrysville, Pennsylvania 15668.

15. Approval Signatures for Amendment B:

Ronald L. Joiner, Ph.D.
Study Director

15 April 1986
Date

H. Hugh Harroff, Jr. D.V.M.
Chief Veterinarian

15 April 1986
Date

LTC(P) Howard C. Johnson, D.V.M.
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15 Apr 86
Date

APPENDIX C

Tables

TABLE 3.1.1. OPTIMIZATION A: RESULTS OF FUNNEL COLLECTION METHOD FOR VOLATILIZED HD AND L

No. Animals	No. Sites Dosed Per Animal	Decontamination System		Decontamination at 4 Hr (Both Sides)	Start of Sample Period* (min)	Volatilized Agent per Animal (ng/l)
		Left	Right			
			HD			
8	7	Water	M258A1 I, II	0.5% NaClO	240	<0.6**
8	7	Water	M258A1 I, II	0.5% NaClO	240	<0.6
8	7	Water	M258A1 I, II	0.5% NaClO	240	<0.6
1	7	Water	M258A1 I, II	None	240	<0.6
1	7	None	None	None	240	<0.6
1	7	None	None	None	7	2,740
1	7	None	None	None	11	880
			L			
8	7	Water	M258A1 I, II	0.5% NaClO	240	<2.4***
8	7	Water	M258A1 I, II	0.5% NaClO	240	<1.14

*Time after HD dosing; sample period duration = 180 min.
**Time weighted average (permissible exposure limits) for HD in air = 3 ng/l; detection limit for HD = 0.20; TWA = 0.6 ng/l.
***Time weighted average (permissible exposure limits) for L = 3.0 ng/l; detection limit for As (5 ng/l) is equivalent to 0.38 TWA for L = 1.14 ng/l.

TABLE 3.1.2. LESION LENGTHS (in mm) FOR 0.5 µl OF HD FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 1.25, 5.0, AND 10.0 MIN AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B3465M	20	23	29	27	37	34	32
1	B3535F	15	22	29	30	35	32	29
1	B3482M	22	24	26	28	34	36	28
1	B3399F	17	32	29	27	41	45	33
1	B3468M	15	24	17	23	21	19	23
1	B3409F	28	24	22	27	44	32	34
1	B3446M	20	23	24	24	29	36	23
1	B3437F	21	22	22	31	28	34	32
2	B3731M	17	22	26	25	61	40	37
2	B3680F	25	24	24	28	42	59	58
2	B3722M	14	20	31	28	39	42	46
2	B3777F	16	18	19	22	47	44	29
2	B3717M	18	28	28	25	34	35	31
2	B3746M	*	*	*	*	*	*	*
2	B3704F	16	20	23	31	23	26	25
2	B3781M	13	23	22	22	23	26	27
3	B3988M	28	24	21	29	32	36	28
3	B3784F	19	24	17	34	29	45	26
3	B4012M	16	22	27	29	45	37	36
3	B4067F	27	23	25	32	12	36	28
3	B3997M	32	31	29	28	47	40	34
3	B4053F	21	19	24	25	12	27	26
3	B3998M	26	31	27	34	37	69	42
3	B4049F	23	23	24	32	40	39	34
3	B3996M	27	25	26	34	51	47	30

*Animal was incorrectly dosed.

TABLE 3.1.3. LESION WIDTHS (in mm) FOR 0.5 µl OF HD FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 1.25, 5.0, AND 10.0 MIN AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B3465M	8	12	13	17	11	14	12
1	B3535F	6	12	10	16	11	11	12
1	B3482M	9	13	15	17	14	11	16
1	B3399F	6	12	14	17	15	14	17
1	B3468M	4	7	8	17	8	7	12
1	B3409F	9	9	10	18	13	13	16
1	B3446M	5	7	11	17	12	10	11
1	B3437F	6	9	13	19	10	14	12
2	B3731M	5	13	16	22	11	14	20
2	B3680F	15	17	16	25	15	17	18
2	B3722M	12	15	19	19	16	19	19
2	B3777F	10	9	14	14	16	16	19
2	B3717M	12	11	13	17	10	13	17
2	B3704F	5	12	11	20	10	11	14
2	B3746M	*	*	*	*	*	*	*
2	B3781M	5	10	13	26	8	14	18
3	B3988M	9	5	6	17	8	14	12
3	B3784F	5	8	9	15	10	16	11
3	B4012M	7	9	15	17	12	13	16
3	B4067F	8	12	14	22	9	14	16
3	B3997M	8	14	13	17	13	15	16
3	B4053F	12	14	11	15	10	12	14
3	B3998M	8	16	17	19	13	15	19
3	B4049F	8	9	11	19	11	12	16
3	B3996M	12	15	13	24	16	17	17

*Animal was incorrectly dosed.

TABLE 3.1.4. LESION AREAS (in mm^2) FOR 0.5 µl OF HD FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 1.25, 5.0, AND 10.0 MIN AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B3465M	126	217	296	360	319	374	301
1	B3535F	71	207	228	377	302	276	273
1	B3482M	155	245	306	374	374	311	352
1	B3399F	80	301	319	360	483	495	440
1	B3468M	47	132	107	307	132	104	217
1	B3409F	198	170	173	382	449	327	427
1	B3446M	79	126	207	320	273	283	199
1	B3437F	99	155	225	462	220	374	301
2	B3731M	67	225	327	432	527	440	581
2	B3680F	294	320	301	550	495	787	820
2	B3722M	132	236	462	418	490	626	686
2	B3777F	126	127	209	242	590	553	433
2	B3717M	170	242	286	334	267	357	414
2	B3704F	63	188	199	487	181	225	275
2	B3746M	*	*	*	*	*	*	*
2	B3781M	51	181	225	449	144	286	382
3	B3988M	198	94	99	387	201	396	264
3	B3784F	75	151	120	400	228	565	225
3	B4012M	88	155	318	387	424	378	452
3	B4067F	170	217	274	553	85	396	352
3	B3997M	201	341	296	374	480	471	427
3	B4053F	198	209	207	294	94	254	286
3	B3998M	163	389	360	507	378	812	626
3	B4049F	144	162	207	477	345	367	427
3	B3996M	254	294	265	641	641	627	400

*Animal was incorrectly dosed.

TABLE 3.1.5. LESION LENGTHS (in mm) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN (TASK 85-11) AGAINST 0.5 µl OF HD

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B1592M	24	24	24	24	26	32	26
1	B1346F	22	24	24	24	22	22	22
1	B1304M	14	20	24	25	34	23	23
1	B1342F	24	20	25	30	25	34	23
1	B1634M	20	25	25	28	38	27	35
1	B1336F	20	16	19	22	26	33	22
1	B1508M	24	30	37	30	40	46	41
1	B1483F	30	28	34	25	40	33	28
2	B1459M	20	22	22	24	38	30	20
2	B1325F	36	35	35	42	36	34	35
2	B1306M	28	34	35	35	40	47	44
2	B1338F	15	25	25	25	28	30	25
2	B1529M	22	35	30	30	50	45	38
2	B1326F	25	30	30	28	55	42	38
2	B1496M	25	34	30	35	58	60	35
2	B1349F	24	28	35	22	35	42	35
3	B1609M	18	20	24	26	47	37	27
3	B1731F	30	27	31	37	40	40	35
3	B1507M	25	35	35	35	30	35	30
3	B1732F	25	37	46	38	35	42	35
3	B1309M	25	38	40	45	50	47	47
3	B1698F	25	37	35	35	50	40	35
3	B1307M	22	32	34	35	45	39	27
3	B1655F	20	25	23	35	32	30	25

TABLE 3.1.6. LESION WIDTHS (in mm) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN (TASK 85-11) AGAINST 0.5 µl OF HD

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B1592M	18	18	16	22	16	20	18
1	B1346F	12	18	16	15	13	10	16
1	B1304M	8	12	15	20	13	11	14
1	B1342F	6	10	13	18	12	16	9
1	B1634M	10	15	18	22	14	17	16
1	B1336F	9	8	11	21	7	8	11
1	B1508M	8	11	14	20	18	17	16
1	B1483F	10	11	15	20	11	11	12
2	B1459M	12	14	15	16	12	14	8
2	B1325F	24	15	18	20	18	18	15
2	B1306M	7	12	14	20	14	15	14
2	B1338F	5	10	10	19	15	14	16
2	B1529M	8	14	15	22	12	17	17
2	B1326F	7	10	10	18	12	14	15
2	B1496M	12	13	12	20	17	15	17
2	B1349F	12	12	15	17	12	13	15
3	B1609M	5	8	10	15	8	12	12
3	B1731F	12	17	20	20	15	20	20
3	B1507M	15	18	17	17	17	18	23
3	B1732F	17	18	17	26	18	20	18
3	B1309M	14	16	17	25	22	17	20
3	B1698F	12	12	15	20	20	20	16
3	B1307M	8	13	14	20	18	18	17
3	B1655F	9	11	15	17	14	10	12

TABLE 3.1.7. LESION AREAS (in mm^2) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN (TASK 85-11) AGAINST 0.5 µl OF HD

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
1	B1592M	339	339	301	414	327	502	367
1	B1346F	207	339	301	283	225	173	276
1	B1304M	88	188	283	393	347	199	253
1	B1342F	113	157	255	424	236	427	162
1	B1634M	157	294	353	484	418	360	440
1	B1336F	141	100	164	363	143	207	190
1	B1508M	151	259	407	471	565	614	515
1	B1483F	236	242	400	393	345	285	264
2	B1459M	188	242	259	301	358	330	126
2	B1325F	678	412	495	659	509	480	412
2	B1306M	154	320	385	550	440	553	484
2	B1338F	59	196	196	373	330	330	314
2	B1529M	138	385	353	518	471	601	507
2	B1326F	137	236	236	396	518	462	447
2	B1496M	236	347	283	550	774	707	467
2	B1349F	226	264	412	294	330	429	412
3	B1609M	71	126	188	306	295	349	254
3	B1731F	283	360	487	581	471	628	550
3	B1507M	294	495	467	467	400	495	542
3	B1732F	334	523	614	776	495	659	495
3	B1309M	275	477	534	883	864	627	738
3	B1698F	236	349	412	550	785	628	440
3	B1307M	138	327	374	550	636	551	360
3	B1655M	141	216	271	467	352	236	236

TABLE 3.1.8. AVERAGE LESION SIZES USING DISTILLED WATER AND M258A1 I AND II STANDARD KIT MATERIAL IN THE RABBIT MODEL SCREEN FOR CANDIDATE DECONTAMINATION SYSTEMS AGAINST 0.5 µl OF HD AT 1.25, 5.0, OR 10.0 MIN FOLLOWED BY DECONTAMINATION WITH SODIUM HYPOCHLORITE SOLUTIONS OF EITHER 0.5 PERCENT AT 4 HR OR 5.0 PERCENT AT 24 HR AFTER DOSING

Lesion Size Response	M258A1 I & II			Non-decontaminated Control	Distilled Water		
	1.25 Min	5.0 Min	10.0 Min		1.25 Min	5.0 Min	10.0 Min
				0.5% Sodium Hypochlorite at 4 Hr After Dosing			
Lengths (mm)	20.7	23.8	24.6	28.1	35.1*	38.2*	32.1*
Widths (mm)	8.1	11.3	12.7	18.6	11.8*	13.6*	15.4*
Areas (sq mm)	135.3	211.9	250.6	411.3	338.3*	420.1*	398.3*
				5.0% Sodium Hypochlorite at 24 Hr After Dosing			
Lengths (mm)	23.5	28.4	30.1	30.6	38.3*	37.1*	31.3
Widths (mm)	10.8	13.2	14.7	19.6	14.5*	15.2*	15.3
Areas (sq mm)	209.1	300.0	351.2	476.8	442.9*	451.2*	385.4

*Significantly different ($P < 0.05$, one-sided) from M258A1 standard decontamination estimates at corresponding time periods.

TABLE 3.1.9. OPTIMIZATION A: EFFECTS ON HD LESION SIZE USING A 4-HR DECONTAMINATION WITH 0.5 PERCENT SODIUM HYPOCHLORITE VERSUS A 24-HR DECONTAMINATION WITH 5.0 PERCENT SODIUM HYPOCHLORITE

Time to Decontamination		4 Hr 0.5% Sodium Hypochlorite				24 Hr 5.0% Sodium Hypochlorite			
		1.25 Min	5.0 Min	10.0 Min	Avg	1.25 Min	5.0 Min	10.0 Min	Avg
Response	Statistic								
Lesion Lengths	Std-Water	-15.0	-14.4	-7.7	-12.3	-15.5	-8.4	-0.7	-8.2
	N	24	24	24	72	24	24	24	72
	SE	1.8	1.8	1.8	1.0	1.5	1.5	1.5	0.9
	Significance	*	*	*	*	*	*	---	*
Lesion Widths	Std-Water	-3.7	-2.3	-2.7	-2.9	-4.0	-2.2	-0.9	-2.4
	N	24	24	24	72	24	24	24	72
	SE	0.6	0.6	0.6	0.4	0.7	0.7	0.7	0.4
	Significance	*	*	*	*	*	*	---	*
Lesion Areas	Std-Water	-207	-208	-151	-189	-254	-154	-34	-148
	N	24	24	24	72	24	24	24	72
	SE	25.6	25.6	25.6	14.8	27.5	27.5	27.5	15.9
	Significance	*	*	*	*	*	*	---	*

Std-Water is the mean difference between contralateral lesion sizes (M258A1 I, II less distilled water).
(Note: Std-Water does not always match the difference between average lesion sizes in Table 3.1.8 due to imbalances in the analysis resulting from nonuniformity in the number of animals used per day across replicates.)
N is the number of animals used.
SE is the standard error of the mean difference.
*Denotes a statistically significant difference ($P < 0.05$, one-sided), i.e., M258A1 I, II is significantly more effective than distilled water.
---Denotes no statistically significant difference ($P \geq 0.05$, one-sided).

TABLE 3.1.10. OPTIMIZATION A: CONTRASTS BETWEEN CONTRALATERAL DIFFERENCES IN LESION SIZE ESTIMATES FROM DECONTAMINATION OF HD AT 4 HR WITH 0.5 PERCENT SODIUM HYPOCHLORITE VERSUS DECONTAMINATION AT 24 HR WITH 5.0 PERCENT SODIUM HYPOCHLORITE

Time to Decontamination		2-Sided t tests			
		1.25 Min	5.0 Min	10.0 Min	Avg
Response	Statistic				
Lesion Lengths	Difference	-0.5	6.0	7.0	4.1
	df	46	46	46	142
	t	-0.2	2.6	3.0	3.0
	Significance	---	*	*	*
Lesion Widths	Difference	-0.3	0.1	1.8	0.5
	df	46	46	46	142
	t	-0.3	0.1	2.0	0.9
	Significance	---	---	---	---
Lesion Areas	Difference	-47.0	54.0	117.0	41.0
	df	46	46	46	142
	t	-1.3	1.4	3.1	1.9
	Significance	---	---	*	---

"Difference" is the contralateral difference for decontamination at 24 hr with 5.0% sodium hypochlorite less the contralateral difference for decontamination at 4 hr with 0.5% sodium hypochlorite.
df is degrees of freedom used to determine significance.
t is the studentized unpaired t statistic.
*Denotes a statistically significant difference ($P < 0.05$, two-sided).
---Denotes no statistically significant difference ($P \geq 0.05$, two-sided).

Therefore, the t value is an index of the degree of improvement in the model due to the early decontamination procedure.

TABLE 3.1.11. LESION LENGTHS (in mm) FOR 0.5 µl OF L FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 30, 60, AND 120 SEC AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5414M	15	15	15	19	21	19	21
1	B5470F	14	15	15	*	13	18	18
1	B5404M	13	19	19	21	18	17	18
1	B5477F	14	15	16	22	17	18	21
1	B5423M	15	16	13	22	21	20	18
1	B5278F	18	17	17	23	19	20	16
1	B5433M	17	18	17	28	19	18	22
1	B5479F	16	19	18	31	19	22	27
2	B5521M	15	19	16	23	18	19	20
2	B5560F	17	21	15	26	20	20	19
2	B5514M	16	21	18	28	20	23	20
2	B5547F	16	18	15	26	20	22	25
2	B5518M	14	15	15	27	18	17	24
2	B5563F	11	11	18	20	20	21	19
2	B5496M	12	17	14	30	19	17	19
2	B5562F	14	18	18	23	20	18	17

*Needle skipped during dosing.

TABLE 3.1.12. LESION WIDTHS (in mm) FOR 0.5 µl OF L FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 30, 60, AND 120 SEC AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5414M	4	6	7	10	6	9	9
1	B5470F	4	5	7	*	4	7	8
1	B5404M	3	4	5	12	5	5	7
1	B5477F	5	5	5	14	6	7	7
1	B5423M	4	4	5	13	5	6	8
1	B5278F	4	5	6	15	6	7	9
1	B5433M	4	5	7	14	6	6	8
1	B5479F	6	6	7	17	8	8	11
2	B5521M	4	5	4	18	6	6	6
2	B5560F	4	3	3	18	6	5	6
2	B5514M	3	4	5	20	5	6	6
2	B5547F	4	5	8	16	5	6	9
2	B5518M	4	5	6	20	6	6	10
2	B5563F	3	4	5	17	4	5	7
2	B5496M	5	4	4	15	5	6	7
2	B5562F	3	3	4	13	4	5	6

*Needle skipped during dosing.

TABLE 3.1.13. LESION AREAS (in mm^2) FOR 0.5 µl OF L FOLLOWED BY M258A1 I AND II OR DISTILLED WATER DECONTAMINATION AT 30, 60, AND 120 SEC AND 0.5 PERCENT SODIUM HYPOCHLORITE DECONTAMINATION AT 4 HR AFTER DOSING

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5414M	47	71	82	149	99	134	148
1	B5470F	44	59	82	*	41	99	113
1	B5404M	31	60	75	198	71	67	99
1	B5477F	55	59	63	242	80	99	115
1	B5423M	47	50	51	225	82	94	113
1	B5278F	57	67	80	271	89	110	113
1	B5433M	53	71	93	308	89	85	138
1	B5479F	75	89	99	414	119	138	233
2	B5521M	47	75	50	325	85	89	94
2	B5560F	53	49	35	367	94	79	89
2	B5514M	38	66	71	440	79	108	94
2	B5547F	50	71	94	327	79	104	177
2	B5518M	44	59	71	424	85	80	188
2	B5563F	26	35	71	267	63	82	104
2	B5496M	47	53	44	353	75	80	104
2	B5562F	33	42	57	235	63	71	80

*Needle skipped during dosing.

TABLE 3.1.14. LESION LENGTHS (in mm) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN AGAINST 0.5 µl OF L

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5032M	23	28	25	24	19	19	24
1	B5084F	18	18	20	24	19	19	19
1	B5011M	23	24	21	21	24	19	20
1	B5051F	22	23	19	24	21	18	22
1	B4801M	22	18	20	21	22	18	20
1	B5067F	19	19	20	21	16	20	24
1	B4811M	18	19	20	29	20	21	23
1	B5071F	17	17	20	23	20	19	19
2	B5010M	18	21	19	28	20	22	20
2	B4773F	19	19	17	27	18	17	18
2	B5017M	19	17	17	21	19	18	22
2	B5061F	20	18	19	28	22	19	20
2	B4818M	17	16	17	32	18	20	20
2	B4860F	21	17	17	20	19	21	18
2	B5014M	15	17	19	25	25	18	20
2	B5068F	15	19	22	22	23	17	15
3	B4798M	22	22	25	28	28	24	25
3	B4754F	17	25	18	22	20	19	18
3	B5035M	19	20	19	22	15	17	17
3	B5089F	17	15	22	22	17	20	18
3	B5020M	13	17	18	21	19	16	20
3	B4869F	15	18	16	24	16	16	20
3	B4802M	13	17	17	24	15	19	18
3	B5054F	*	12	15	26	15	15	19

*Needle skipped during dosing.

TABLE 3.1.15. LESION WIDTHS (in mm) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN AGAINST 0.5 µl OF L

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5032M	12	7	9	17	9	9	11
1	B5084F	4	6	6	22	6	7	9
1	B5011M	5	8	8	18	7	8	8
1	B5051F	11	10	7	23	6	8	10
1	B4801M	9	9	9	17	8	7	7
1	B5067F	5	5	7	22	6	8	11
1	B4811M	8	7	8	21	9	9	10
1	B5071F	4	5	7	19	7	8	9
2	B5010M	4	8	6	25	7	8	10
2	B4773F	5	5	6	19	8	9	10
2	B5017M	8	7	8	17	8	6	7
2	B5061F	6	5	6	17	8	7	7
2	B4818M	4	5	4	20	7	7	8
2	B4860F	8	9	6	14	6	6	9
2	B5014M	6	6	5	20	6	7	8
2	B5068F	7	7	8	13	8	8	8
3	B4798M	9	9	10	20	10	9	10
3	B4754F	5	5	6	15	12	10	10
3	B5035M	6	7	10	17	7	8	10
3	B5089F	7	6	10	13	7	10	9
3	B5020M	5	7	9	13	6	5	7
3	B4869F	12	6	6	17	12	9	11
3	B4802M	5	6	5	15	7	7	8
3	B5054F	*	4	4	17	8	8	8

*Needle skipped during dosing.

TABLE 3.1.16. LESION AREAS (in mm^2) USING M258A1 I AND II DECONTAMINATION SYSTEM VERSUS DISTILLED WATER IN THE MREF PROTOCOL 22 SCREEN AGAINST 0.5 µl OF L

Replicate	Animal Number	M258A1 I & II			Non-decontaminated Control	Distilled Water		
		30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
1	B5032M	217	154	177	320	134	134	207
1	B5084F	57	85	94	414	89	104	134
1	B5011M	90	151	132	297	132	119	126
1	B5051F	190	181	104	433	99	113	173
1	B4801M	155	127	141	280	138	99	110
1	B5067F	75	75	110	363	75	126	207
1	B4811M	113	104	126	478	141	148	181
1	B5071F	53	67	110	343	110	119	134
2	B5010M	57	132	89	550	110	138	157
2	B4773F	75	75	80	403	113	120	141
2	B5017M	119	93	107	280	119	85	121
2	B5061F	94	71	89	374	138	104	110
2	B4818M	53	63	53	502	99	110	126
2	B4860F	132	120	80	220	89	99	127
2	B5014M	71	80	75	393	118	99	126
2	B5068F	82	104	138	225	144	107	94
3	B4798M	155	155	196	440	220	170	196
3	B4754F	67	98	85	259	188	149	141
3	B5035M	89	110	149	294	82	107	133
3	B5089F	93	71	173	225	93	157	127
3	B5020M	51	93	127	214	89	63	110
3	B4869F	141	85	75	320	151	113	173
3	B4802M	51	80	67	283	82	104	113
3	B5054F	*	38	47	347	94	94	119

*Needle skipped during dosing.

TABLE 3.1.17. AVERAGE LESION SIZES USING DISTILLED WATER AND M258A1 I AND II STANDARD KIT MATERIAL IN THE RABBIT MODEL SCREEN FOR CANDIDATE DECONTAMINATION SYSTEMS AGAINST 0.5 µl of L at 30, 60, AND 120 SEC FOLLOWED BY DECONTAMINATION WITH SODIUM HYPOCHLORITE SOLUTIONS OF EITHER 0.5 PERCENT AT 4 HR OR 5.0 PERCENT AT 24 HR AFTER DOSING

Lesion Size Response	M258A1 I & II			Non-decontaminated Control	Distilled Water		
	30 Sec	60 Sec	120 Sec		30 Sec	60 Sec	120 Sec
				0.5% Sodium Hypochlorite at 4 Hr After Dosing			
Lengths (mm)	14.8	17.1	16.2	24.6	18.9*	19.3*	20.3*
Widths (mm)	4.0	4.6	5.5	15.5	5.4*	6.3*	7.8*
Areas (sq mm)	46.7	60.9	69.9	302.9	80.8*	94.9*	125.3*
				5.0% Sodium Hypochlorite at 24 Hr After Dosing			
Lengths (mm)	18.3	19.0	19.3	24.1	19.6*	18.8	20.0
Widths (mm)	6.7	6.6	7.1	18.0	7.7*	7.8*	9.0*
Areas (sq mm)	99.2	100.4	109.4	344.0	118.8*	115.9	141.1*

*Significantly different ($P < 0.05$, one-sided) from M258A1 standard decontamination estimates at corresponding time periods.

TABLE 3.1.18. OPTIMIZATION A: EFFECTS ON L LESION SIZE USING A 4-HR DECONTAMINATION WITH 0.5 PERCENT SODIUM HYPOCHLORITE VERSUS A 24-HR DECONTAMINATION WITH 5.0 PERCENT SODIUM HYPOCHLORITE

Time to Decontamination		4 Hr 0.5% Sodium Hypochlorite				24 Hr 5.0% Sodium Hypochlorite			
		30 Sec	60 Sec	120 Sec	Avg	30 Sec	60 Sec	120 Sec	Avg
Response	Statistic								
Lesion Lengths	Std-Water	-4.1	-2.2	-4.1	-3.4	-1.5	0.2	-0.7	-0.7
	N	16	16	16	48	23	24	24	71
	SE	0.8	0.8	0.8	0.45	0.8	0.7	0.7	0.4
	Significance	*	*	*	*	*	---	---	---
Lesion Widths	Std-Water	-1.4	-1.7	-2.3	-1.8	-0.5	-1.4	-1.8	-1.5
	N	16	16	16	48	23	24	24	71
	SE	0.4	0.4	0.4	0.2	0.6	0.6	0.6	0.3
	Significance	*	*	*	*	*	*	*	*
Lesion Areas	Std-Water	-34.0	-34.0	-55.4	-41.2	-24.8	-18.7	-29.9	-24.4
	N	16	16	16	48	23	24	24	71
	SE	10.6	10.6	10.6	6.1	13.4	13.0	13.0	7.6
	Significance	*	*	*	*	*	---	*	*

Std-Water is the mean difference between contralateral lesion sizes (M258A1 I, II less distilled water).
(Note: Std-Water does not always match the difference between average lesion sizes in Table 3.1.17 due to imbalances in the analysis resulting from occasional missing data.)
N is the number of animals used.
SE is the standard error of the mean difference.
*Denotes a statistically significant difference ($P < 0.05$, one-sided), i.e., M258A1 I, II is significantly more effective than distilled water.
---Denotes no statistically significant difference ($P \geq 0.05$, one-sided).

TABLE 3.1.19. OPTIMIZATION A: CONTRASTS BETWEEN CONTRALATERAL DIFFERENCES IN LESION SIZE ESTIMATES FROM DECONTAMINATION OF L AT 4 HR WITH 0.5 PERCENT SODIUM HYPOCHLORITE VERSUS DECONTAMINATION AT 24 HR WITH 5.0 PERCENT SODIUM HYPOCHLORITE

Time to Decontamination		2-Sided t tests			
		30 Sec	60 Sec	120 Sec	Avg
Response	Statistic				
Lesion Lengths	Difference	2.6	2.4	3.4	2.7
	df	37	38	38	63
	t	2.2	2.2	3.2	2.5
	Significance	*	*	*	*
Lesion Widths	Difference	0.2	0.3	0.5	0.3
	df	36	37	37	113
	t	1.2	0.4	0.7	0.8
	Significance	---	---	---	---
Lesion Areas	Difference	9.2	15.3	25.5	16.8
	df	37	38	38	117
	t	0.5	0.8	1.4	1.7
	Significance	---	---	---	---

"Difference" is the contralateral difference for decontamination at 24 hr with 5.0% sodium hypochlorite less the contralateral difference for decontamination at 4 hr with 0.5% sodium hypochlorite.
df is degrees of freedom used to determine significance.
t is the studentized unpaired t statistic.
*Denotes a statistically significant difference ($P < 0.05$, two-sided).
---Denotes no statistically significant difference ($P \geq 0.05$, two-sided).

Therefore, the t value is an index of the degree of improvement in the model due to the early decontamination procedure.

TABLE 3.2.1. GROUP 1 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR ORIGINAL TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		1.25 Min	5.0 Min	10.0 Min	24 Hr	1.25 Min	5.0 Min	10.0 Min	24 Hr
Lengths	B3545M	24	24	22	25	19	22	25	26
	B3509F	15	27	19	19	12	24	22	18
	B3546M	15	21	22	16	20	20	21	23
	B3702F	17	18	20	17	14	18	14	19
	B3667M	11	17	19	19	13	15	16	24
	B3679F	15	15	18	26	20	20	15	31
	B3656M	17	18	21	24	16	16	22	22
	B3599F	13	16	14	18	13	16	18	18
Widths	B3545M	11	10	12	9	5	8	14	16
	B3509F	5	11	9	13	5	8	10	20
	B3546M	6	9	10	12	7	11	16	19
	B3702F	3	6	6	12	3	6	8	9
	B3667M	10	5	8	9	6	6	8	10
	B3679F	2	4	10	20	3	5	8	20
	B3656M	2	7	7	15	3	7	9	11
	B3599F	4	6	7	7	4	6	7	8
Areas	B3545M	207	188	207	177	75	138	275	327
	B3509F	59	233	134	194	47	151	173	283
	B3546M	71	148	173	151	110	173	264	343
	B3702F	40	85	94	160	33	85	88	134
	B3667M	86	67	119	134	61	71	100	188
	B3679F	24	47	141	408	47	79	94	487
	B3656M	27	99	115	283	38	88	155	190
	B3599F	41	75	77	99	41	75	99	113

TABLE 3.2.2. GROUP 2 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR ORIGINAL TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		5.0 Min	10.0 Min	24 Hr	1.25 Min	5.0 Min	10.0 Min	24 Hr	1.25 Min
Lengths	B3574M	24	22	24	14	20	22	24	15
	B3587F	17	15	24	9	18	11	21	15
	BOM*	14	19	25	12	15	22	26	18
	B3610F	14	16	15	14	16	15	26	16
	B3649M	14	19	29	11	16	23	26	15
	B3612F	14	21	29	16	19	19	30	16
	B3661M	21	18	19	15	17	19	24	16
	B3585F	14	16	15	13	17	19	20	12
Widths	B3574M	14	12	16	4	8	9	15	3
	B3587F	7	8	11	5	4	7	9	4
	BOM*	5	6	11	3	6	5	12	5
	B3610F	8	6	15	3	6	6	12	3
	B3649M	7	10	18	8	7	13	15	7
	B3612F	7	12	16	5	7	9	17	6
	B3661M	5	5	17	3	5	4	16	3
	B3585F	4	7	6	4	7	10	10	5
Areas	B3574M	264	207	301	44	126	155	283	35
	B3587F	93	94	207	35	57	60	148	47
	BOM*	55	89	216	28	71	86	245	71
	B3610F	88	75	177	33	75	71	245	38
	B3649M	77	149	410	69	88	235	306	82
	B3612F	77	198	364	63	104	134	400	75
	B3661M	82	71	254	35	67	60	301	38
	B3585F	44	88	71	41	93	149	157	47

*Unreadable tatoo; number BOM assigned

TABLE 3.2.3. GROUP 3 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR ORIGINAL TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		10.0 Min	24 Hr	1.25 Min	5.0 Min	10.0 Min	24 Hr	1.25 Min	5.0 Min
Lengths	B3560M	22	24	15	17	24	20	17	20
	B3607F	17	25	*	15	16	20	20	25
	B3451M	21	25	15	19	20	20	18	23
	B3685F	19	25	16	24	25	26	26	20
	B3644M	20	36	15	24	16	32	14	28
	B3614F	18	21	12	11	23	20	14	16
	B3627M	17	15	15	15	17	18	16	18
	B3674F	15	22	12	11	19	23	15	*
Widths	B3560M	14	15	3	7	11	11	5	13
	B3607F	11	13	*	8	19	11	5	7
	B3451M	11	14	5	10	12	11	4	8
	B3685F	13	20	5	10	17	15	11	9
	B3644M	7	19	7	8	6	21	4	18
	B3614F	14	15	5	8	11	15	5	6
	B3627M	6	7	2	4	6	9	3	4
	B3674F	8	15	4	6	13	18	4	*
Areas	B3560M	242	283	35	93	207	173	67	204
	B3607F	147	255	*	94	239	173	79	137
	B3451M	181	275	59	149	188	173	57	144
	B3685F	194	392	63	188	334	306	225	141
	B3644M	110	537	82	151	75	528	44	396
	B3614F	198	247	47	69	199	236	55	75
	B3627M	80	82	24	47	80	127	38	57
	B3674F	94	259	38	52	194	325	47	*

TABLE 3.2.4. GROUP 4 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR ORIGINAL TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination 24 Hr	1.25 Min	5.0 Min	10.0 Min	Time to Decontamination 24 Hr	1.25 Min	5.0 Min	10.0 Min
Lengths	B3484M	20	15	17	22	22	18	20	17
	B3684F	18	15	17	30	31	10	20	20
	B3619M	19	15	18	18	20	20	27	18
	B3593F	23	18	21	19	23	18	19	22
	B3622M	26	15	13	19	28	*	15	14
	B3608F	23	14	16	15	20	***	19	20
	B3621M	26	14	13	21	25	15	**	22
	B3590F	28	17	16	17	24	13	13	14
Widths	B3484M	18	4	7	8	19	3	5	6
	B3684F	13	4	5	18	25	2	4	7
	B3619M	10	3	5	6	10	4	4	7
	B3593F	13	5	8	10	13	5	11	11
	B3622M	25	4	7	6	10	*	6	9
	B3608F	15	3	4	8	17	***	4	8
	B3621M	34	3	6	9	23	4	**	10
	B3590F	17	4	6	7	15	4	6	7
Areas	B3484M	283	47	93	138	328	42	79	80
	B3684F	184	47	67	424	608	16	63	110
	B3619M	149	35	71	85	157	63	85	99
	B3593F	235	71	132	149	235	71	164	190
	B3622M	510	47	71	89	220	*	71	99
	B3608F	271	33	50	94	267	***	60	126
	B3621M	694	33	61	148	451	47	**	173
	B3590F	374	53	75	93	283	41	61	77

*M258A1 burn too extensive to read lesion.
**Site not deconned at correct time.
***Needle skipped during dosing.

TABLE 3.2.5. GROUP 1 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr
Lengths	B4418M	18	22	22	30	17	22	22	34
	B4375F	18	20	20	23	26	22	22	24
	B4402M	19	25	24	32	23	25	25	30
	B4371F	18	22	23	30	17	22	22	33
	B4505M	20	23	26	28	17	21	20	27
	B4464F	20	21	20	25	18	17	22	26
	B4434M	22	26	22	24	20	23	22	25
	B4476F	21	26	23	24	19	22	25	25
Widths	B4418M	5	7	12	22	4	8	15	22
	B4375F	4	6	6	8	5	4	8	10
	B4402M	8	15	15	19	9	10	15	21
	B4371F	5	9	8	19	8	11	12	17
	B4505M	13	12	15	21	8	10	10	20
	B4464F	8	9	13	19	5	8	11	18
	B4434M	5	9	9	18	4	5	9	17
	B4476F	9	12	14	17	5	8	9	23
Areas	B4418M	71	121	207	518	53	138	259	587
	B4375F	57	94	94	144	102	69	138	188
	B4402M	119	294	283	477	162	196	294	495
	B4371F	71	155	144	447	107	190	207	440
	B4505M	204	217	306	462	107	165	157	424
	B4464F	126	148	204	373	71	107	190	367
	B4434M	86	184	155	339	63	90	155	334
	B4476F	148	245	253	320	75	138	177	451

TABLE 3.2.6. GROUP 2 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min
Lengths	B4322M	20	23	33	15	16	20	28	15
	B4340F	24	23	35	26	*	29	30	24
	B4416M	21	25	22	17	22	32	24	18
	B4382F	18	20	16	17	19	25	17	20
	B4529M	20	25	30	21	21	22	34	19
	B4555F	24	23	32	25	23	22	31	15
	B4491M	21	22	26	18	23	26	26	16
	B4475F	14	18	24	18	14	20	23	13
Widths	B4322M	10	10	15	8	7	8	17	5
	B4340F	10	13	15	10	*	13	14	5
	B4416M	12	13	17	9	11	12	15	6
	B4382F	5	5	10	4	7	6	12	6
	B4529M	6	9	22	5	6	6	20	4
	B4555F	11	12	20	6	10	10	22	5
	B4491M	9	11	19	7	10	10	23	7
	B4475F	7	5	24	8	10	9	19	6
Areas	B4322M	157	181	389	94	88	126	374	59
	B4340F	188	235	412	204	*	296	330	94
	B4416M	198	255	294	120	190	301	283	85
	B4382F	71	79	126	53	104	118	160	94
	B4529M	94	177	518	82	99	104	534	60
	B4555F	207	217	502	118	181	173	535	59
	B4491M	148	190	388	99	181	204	469	88
	B4475F	77	71	452	113	110	141	343	61

*Needle skipped during dosing.

TABLE 3.2.7. GROUP 3 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min
Lengths	B4323M	21	22	17	20	16	27	30	18
	B4374F	15	21	15	18	16	20	20	15
	B4401M	22	22	13	22	20	24	22	15
	B4447F	17	25	20	13	16	27	17	17
	B4533M	25	25	23	25	20	28	20	14
	B4536F	20	30	21	19	19	30	17	19
	B4520M	25	29	22	24	24	30	17	18
	B4538F	21	26	19	21	18	30	17	19
Widths	B4323M	9	13	5	10	12	17	7	6
	B4374F	6	15	4	5	6	18	5	5
	B4401M	14	17	9	12	11	18	8	9
	B4447F	9	20	5	7	8	18	4	5
	B4533M	10	18	7	9	10	19	5	8
	B4536F	12	21	7	7	8	22	4	6
	B4520M	10	20	7	9	14	19	5	9
	B4538F	9	20	5	9	8	21	4	9
Areas	B4323M	148	225	67	157	151	360	165	85
	B4374F	71	247	47	71	75	283	79	59
	B4401M	242	294	92	207	173	339	138	106
	B4447F	120	393	79	71	100	382	53	67
	B4533M	196	353	126	177	157	418	79	88
	B4536F	188	495	115	104	119	518	53	89
	B4520M	196	455	121	170	264	447	67	127
	B4538F	148	408	75	148	113	495	53	134

TABLE 3.2.C. GROUP 4 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF HD DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min
Lengths	B4430M	27	22	18	19	24	20	19	20
	B4379F	22	16	17	15	23	19	18	20
	B4332M	24	16	16	15	23	16	17	15
	B4373F	24	12	10	10	26	11	22	15
	B4489M	24	20	18	18	25	18	23	22
	B4457F	28	19	23	20	27	17	18	23
	B4517M	28	21	22	23	30	17	20	20
	B4458F	22	15	17	17	21	16	20	15
Widths	B4430M	24	4	5	9	20	4	5	8
	B4379F	11	3	5	7	12	4	4	5
	B4332M	11	4	5	6	12	3	5	5
	B4373F	15	4	3	3	18	4	5	5
	B4489M	19	5	8	10	19	4	7	8
	B4457F	19	4	7	9	17	4	5	8
	B4517M	19	6	13	10	18	5	6	8
	B4458F	15	3	4	6	15	3	4	6
Areas	B4430M	509	69	71	134	377	63	75	126
	B4379F	190	38	67	82	217	60	57	79
	B4332M	207	50	63	71	217	38	67	59
	B4373F	283	38	24	24	367	35	86	59
	B4489M	358	79	113	141	373	57	126	138
	B4457F	418	60	126	141	360	53	71	144
	B4517M	418	99	225	181	424	67	94	126
	B4458F	259	35	53	80	247	38	63	71

TABLE 3.2.9. MEAN LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT 1.25, 5.0, AND 10.0 MIN AND 24 HR AFTER DOSING

Position	Time	Lengths (mm) Side		Widths (mm) Side		Areas (mm^2) Side	
		Left	Right	Left	Right	Left	Right
1	1.25 Min	15.9	15.9	4.5	5.4	56.4	69.3
	5.0 Min	17.3	16.5	6.3	7.1	85.1	97.5
	10.0 Min	20.0	18.6	11.9	10.5	189.5	155.7
	24 Hr	24.1	22.9	16.5	18.1	318.6	337.4
2	1.25 Min	15.7	15.4	3.7	3.8	46.6	45.8
	5.0 Min	18.9	19.5	7.1	7.3	107.3	117.8
	10.0 Min	18.8	18.3	7.9	8.3	118.8	121.5
	24 Hr	22.4	24.1	13.9	14.8	254.9	291.3
3	1.25 Min	17.5	14.3	5.1	4.4	76.2	49.7
	5.0 Min	19.0	16.4	5.7	6.0	83.1	77.6
	10.0 Min	19.1	19.4	10.0	8.6	156.0	132.7
	24 Hr	24.6	22.5	13.3	13.8	260.7	249.9
4	1.25 Min	15.4	13.0	4.5	4.4	54.2	43.6
	5.0 Min	21.4	17.0	9.3	7.6	165.0	105.5
	10.0 Min	18.4	20.0	8.1	9.0	119.1	152.7
	24 Hr	22.6	20.5	14.1	12.1	258.1	200.7

TABLE 3.2.10. MEAN LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT 1.0, 3.0, AND 5.0 MIN AND 24 HR AFTER DOSING

Position	Time	Lengths (mm) Side		Widths (mm) Side		Areas (mm^2) Side	
		Left	Right	Left	Right	Left	Right
1	1.0 Min	19.6	19.5	6.0	7.1	92.4	110.2
	3.0 Min	19.7	20.3	8.7	8.8	136.0	142.6
	5.0 Min	18.6	20.8	9.6	9.9	144.0	163.8
	24 Hr	24.9	24.9	16.4	16.6	322.7	330.1
2	1.0 Min	16.8	17.6	3.9	4.1	51.1	58.4
	3.0 Min	21.8	23.1	8.0	9.9	136.7	182.3
	5.0 Min	24.5	22.4	9.3	9.8	182.8	175.3
	24 Hr	27.0	25.0	19.0	18.0	405.2	358.6
3	1.0 Min	20.0	18.8	5.3	6.1	85.9	90.2
	3.0 Min	19.6	17.6	5.1	6.3	79.8	92.6
	5.0 Min	22.5	22.5	11.1	11.5	197.2	205.9
	24 Hr	26.6	27.3	17.8	17.8	378.5	385.0
4	1.0 Min	17.5	19.6	5.5	7.1	75.0	110.5
	3.0 Min	16.9	20.3	7.1	8.5	94.4	138.2
	5.0 Min	18.8	17.1	6.6	7.5	100.1	106.8
	24 Hr	28.0	27.0	18.5	17.9	410.8	385.1

TABLE 3.2.11. MEAN LESION ESTIMATES AVERAGED ACROSS TIME TO DECONTAMINATION BY ANTERIOR-POSTERIOR POSITION AND SIDE FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I and II AT ORIGINAL AND VALIDATED TIMES TO DECONTAMINATION

Original Times to Decontamination: 1.25, 5.0, and 10.0 Min and 24 Hr

	Lengths (mm)			Widths (mm)			Areas (mm^2)		
Position	Left	Right	Avg.	Left	Right	Avg.	Left	Right	Avg.
1	19.3	18.5	18.9	9.8	10.3	10.0	162.4	165.0	163.7
2	19.1	19.3	19.2	8.4	8.5	8.5	137.6	144.1	141.0
3	20.1	18.3	19.2	8.6	8.3	8.5	146.0	130.0	138.0
4	19.4	17.7	18.5	9.0	8.3	8.6	148.6	125.6	136.9
Avg.	19.5	18.4	18.9	9.0	8.9	8.9	148.8	141.3	145.0

Validated Times to Decontamination: 1.0, 3.0, and 5.0 Min and 24 Hr

	Lengths (mm)			Widths (mm)			Areas (mm^2)		
Position	Left	Right	Avg.	Left	Right	Avg.	Left	Right	Avg.
1	20.7	21.3	21.0	10.2	10.6	10.4	175.0	186.7	180.9
2	22.5	22.0	22.3	10.0	10.4	10.2	193.9	193.7	193.8
3	22.2	21.5	21.9	9.8	10.4	10.1	185.3	193.4	189.4
4	20.3	21.0	20.6	9.4	10.3	9.8	170.1	185.1	177.6
Avg.	21.4	21.5	21.5	9.9	10.4	10.1	181.1	189.7	185.4

TABLE 3.2.12. EFFECTS OF ANTERIOR-POSTERIOR POSITION, SIDE, TIME TO DECONTAMINATION, AND INTERACTIONS UPON LESION SIZE ESTIMATES FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT ORIGINAL OR AT VALIDATED TIMES TO DECONTAMINATION

Effect (Source of Variation)	Degrees of Freedom	Significance: Times at 1.25, 5.0, and 10.0 Min and 24 Hr			Significance: Times at 1.0, 3.0, and 5.0 Min and 24 Hr		
		Lengths	Widths	Areas	Lengths	Widths	Areas
Position	3	---	**	---	**	---	---
Side	1	*	---	---	---	*	---
Time	3	***	***	***	***	***	***
Position x Side	3	---	---	---	---	---	---
Position x Time	6	---	*	*	---	---	---
Side x Time	3	---	---	---	---	---	---
Position x Side x Time	9	---	---	---	---	---	---

*Denotes statistical significance for the effect at alpha = 0.05.
**Denotes statistical significance for the effect at alpha = 0.01.
***Denotes statistical significance for the effect at alpha = 0.001.
---Denotes no statistical significance ($P \geq 0.05$).

TABLE 3.2.13. TRENDS AND TWO-SAMPLE CONTRASTS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT ORIGINAL OR AT VALIDATED TIMES TO DECONTAMINATION

	Significance					
	Times at 1.25, 5.0, and 10.0 Min and 24 Hr			Times at 1.0, 3.0, and 5.0 Min and 24 Hr		
	Lengths	Widths	Areas	Lengths	Widths	Areas
Trends						
Estimates Averaged Over Sides and Times for Linear Fit With Positions	---	*	*	---	---	---
Estimates Averaged Over Sides and Times for Quadratic Fit With Positions	---	**	---	**	---	---
Estimates Averaged Over Positions and Sides for Linear Fit With Times	***	***	***	***	***	***
Contrasts: Contralateral Difference Averaged ...						
At Position 1 Over All Times	---	---	---	---	---	---
At Position 2 Over All Times	---	---	---	---	---	---
At Position 3 Over All Times	*	---	---	---	---	---
At Position 4 Over All Times	*	---	---	---	---	---
Over All Positions and Times	*	---	---	---	*	---
For Linear Fit With Positions	---	---	---	---	---	---
For Quadratic Fit With Positions	---	---	---	---	---	---
At Time No. 1 Over All Positions	---	---	---	---	---	---
At Time No. 2 Over All Positions	*	---	---	---	*	---
At Time No. 3 Over All Positions	---	---	---	---	---	---
At Time 24 Hr Over All Positions	---	---	---	---	---	---
For Linear Fit With Times	---	---	---	---	---	---
For Quadratic Fit With Times	---	---	---	---	---	---

*Denotes statistical significance for the trend or for the two-sample contrast at alpha = 0.05.
**Denotes statistical significance for the trend or for the two-sample contrast at alpha = 0.01.
***Denotes statistical significance for the trend or for the two-sample contrast at alpha = 0.001.
---Denotes no statistical significance ($P \geq 0.05$).

TABLE 3.2.14. GROUP 1 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF L DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr
Lengths	B5137M	16	9	13	28	14	14	14	34
	B5157F	20	18	19	24	19	16	16	27
	B5096M	20	20	18	25	17	16	15	26
	B5112F	15	17	20	28	17	21	17	30
	B5295M	24	21	23	*	18	21	18	27
	B5359F	18	20	18	26	19	19	19	18
	B5343M	18	20	16	26	15	15	17	20
	B5394F	17	18	20	29	21	19	22	28
Widths	B5137M	5	4	3	18	5	5	5	16
	B5157F	4	5	4	20	3	4	5	22
	B5096M	5	5	6	21	5	4	5	17
	B5112F	5	5	4	15	4	4	5	20
	B5295M	7	6	10	*	4	5	7	22
	B5359F	3	4	4	16	4	5	5	15
	B5343M	7	4	4	16	2	5	5	15
	B5394F	4	4	3	18	3	4	6	17
Areas	B5137M	63	28	31	396	55	55	55	427
	B5157F	63	71	60	377	45	50	63	466
	B5096M	79	79	85	412	67	50	59	347
	B5112F	59	67	63	330	53	66	67	471
	B5295M	132	99	181	*	57	82	99	466
	B5359F	42	63	57	327	60	75	75	212
	B5343M	99	63	50	327	24	59	67	236
	B5394F	53	57	47	410	49	60	104	374

*Not fully dosed.

TABLE 3.2.15. GROUP 2 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF L DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr	30 Sec
Lengths	B5138M	16	17	20	18	15	19	26	15
	B5165F	18	24	25	15	22	18	26	16
	B5097M	19	18	26	16	15	20	22	15
	B5158F	15	18	26	21	16	16	20	18
	B5331M	20	17	20	16	17	18	25	18
	B5367F	18	19	25	17	18	20	25	19
	B5302M	18	20	25	17	26	21	27	18
	B5355F	18	20	30	22	20	22	24	23
Widths	B5138M	4	4	9	3	4	3	12	3
	B5165F	4	5	15	4	5	5	10	3
	B5097M	5	5	13	6	5	6	19	6
	B5158F	5	5	15	4	4	4	15	4
	B5331M	4	5	13	3	5	5	16	4
	B5367F	5	4	10	4	4	4	7	4
	B5302M	5	3	12	3	4	4	12	2
	B5355F	4	4	13	3	4	4	17	4
Areas	B5138M	50	53	141	42	47	45	245	35
	B5165F	57	94	294	47	86	71	204	38
	B5097M	75	71	265	75	59	94	328	71
	B5158F	59	71	306	66	50	50	236	57
	B5331M	63	67	204	38	67	71	314	57
	B5367F	71	60	196	53	57	63	137	60
	B5302M	71	47	236	40	82	66	254	28
	B5355F	57	63	306	52	63	69	320	72

TABLE 5.2.16. GROUP 5 LESION LENGTHS (IN MM), WIDTHS (IN MM), AND AREAS (IN MM^2) FROM A STUDY TO DETERMINE THE EFFECTS OF L DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Anterior-Posterior Position	Animal's Side	Right				Left			
		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec
Lengths	B5117M	18	23	16	17	18	25	18	17
	B5184F	18	24	22	17	19	*	15	16
	B5136M	20	26	16	17	21	25	15	20
	B5189F	18	27	20	23	18	22	15	18
	B5316M	20	25	17	20	17	25	20	15
	B5377F	17	27	20	18	21	27	16	22
	B5320M	18	24	21	23	19	23	21	23
	B5357F	18	29	17	18	20	26	19	22
Widths	B5117M	5	18	4	5	5	14	4	5
	B5184F	6	14	8	5	6	*	4	5
	B5136M	5	14	3	3	4	12	3	4
	B5189F	5	18	5	5	5	13	3	7
	B5316M	5	15	3	3	4	13	4	4
	B5377F	5	17	3	4	6	17	3	6
	B5320M	6	12	3	4	4	12	3	4
	B5357F	5	14	3	4	5	18	4	5
Areas	B5117M	71	325	50	67	71	275	57	67
	B5184F	85	264	138	67	89	*	47	63
	B5136M	79	286	38	40	66	236	35	63
	B5189F	71	382	79	90	71	225	35	99
	B5316M	79	294	40	47	53	255	63	47
	B5377F	67	360	47	57	99	360	38	104
	B5320M	85	226	49	72	60	217	49	72
	B5357F	71	319	40	57	79	367	60	86

*Decon was spread into area prior to dosing.

TABLE 3.2.17. GROUP 4 LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FROM A STUDY TO DETERMINE THE EFFECTS OF L DOSE POSITION AT FOUR VALIDATED TIMES TO DECONTAMINATION

Animal's Side		Right				Left			
Anterior-Posterior Position		1	2	3	4	1	2	3	4
Lesion Size Response	Animal Number	Time to Decontamination				Time to Decontamination			
		24 Hr	30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec
Lengths	B5129M	28	15	21	18	28	24	17	18
	B5152F	25	16	17	17	22	18	18	17
	B5100M	30	16	19	17	26	15	15	18
	B5169F	27	17	20	17	27	17	22	18
	B5339M	27	19	16	20	30	23	21	19
	B5353F	31	18	20	21	33	21	20	19
	B5300M	33	17	20	23	32	22	22	24
	B5379F	30	19	24	21	22	17	18	20
Widths	B5129M	16	4	7	6	18	3	5	6
	B5152F	20	5	7	6	17	4	5	6
	B5100M	18	4	5	8	17	5	5	6
	B5169F	17	4	4	8	17	4	5	6
	B5339M	18	3	4	6	17	3	5	5
	B5353F	17	3	4	4	14	4	5	6
	B5300M	14	5	4	5	20	3	5	6
	B5379F	18	3	4	5	25	3	4	5
Areas	B5129M	352	47	115	85	396	57	67	85
	B5152F	393	63	93	80	294	57	71	80
	B5100M	424	50	75	107	347	59	59	85
	B5169F	360	53	63	107	360	53	86	85
	B5339M	382	45	50	94	400	54	82	75
	B5353F	414	42	63	66	363	66	79	89
	B5300M	363	67	63	90	502	52	86	113
	B5379F	424	45	75	82	432	40	57	79

TABLE 3.2.18. MEAN LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC AND 24 HR

Position	Time	Lengths (mm) Side		Widths (mm) Side		Areas (mm^2) Side	
		Left	Right	Left	Right	Left	Right
1	30 Sec	17.5	18.5	3.8	5.0	51.1	73.7
	60 Sec	18.6	17.8	4.4	4.5	63.8	62.6
	120 Sec	19.1	18.4	4.9	5.3	73.4	75.7
	24 Hr	27.5	28.9	18.1	17.3	386.7	388.8
2	30 Sec	19.6	17.1	3.6	3.9	54.7	51.5
	60 Sec	17.6	17.9	4.5	4.6	62.1	65.6
	120 Sec	19.3	19.1	4.4	4.4	66.0	65.6
	24 Hr	24.7	25.6	14.1	15.3	276.3	306.9
3	30 Sec	17.4	18.6	3.5	4.0	48.0	60.2
	60 Sec	19.1	19.6	4.9	4.9	73.3	74.7
	120 Sec	17.3	18.4	5.4	4.8	73.4	71.5
	24 Hr	24.4	24.6	13.5	12.5	254.8	243.6
4	30 Sec	17.8	17.8	3.8	3.8	52.1	51.7
	60 Sec	19.1	19.1	5.0	4.1	75.1	62.0
	120 Sec	19.1	19.3	5.8	6.0	86.3	88.9
	24 Hr	26.3	26.6	18.0	17.7	374.8	368.2

TABLE 3.2.19. MEAN LESION ESTIMATES AVERAGED ACROSS TIME TO DECONTAMINATION BY ANTERIOR-POSTERIOR POSITION AND SIDE FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC AND 24 HR

	Lengths (mm)			Widths (mm)			Areas (mm^2)		
Position	Left	Right	Avg.	Left	Right	Avg.	Left	Right	Avg.
1	20.7	20.9	20.8	7.8	8.0	7.9	143.8	150.2	147.0
2	20.2	19.9	20.0	6.4	7.0	6.7	109.6	122.4	116.1
3	19.5	20.3	19.9	6.8	6.5	6.7	112.4	112.5	112.4
4	20.6	20.5	20.5	8.1	7.6	7.9	147.1	135.4	141.3
Avg.	20.2	20.4	20.3	7.3	7.3	7.3	128.3	130.1	129.2

TABLE 3.2.20. EFFECTS OF ANTERIOR-POSTERIOR POSITION, SIDE, TIME TO DECONTAMINATION, AND INTERACTIONS UPON LESION SIZE ESTIMATES FOR 0.5 μl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC AND 24 HR

Effect (Source of Variation)	Degrees of Freedom	Significance Times at 30, 60, and 120 Sec, and 24 Hr		
		Lengths	Widths	Areas
Position	3	---	***	***
Side	1	---	---	---
Time	3	***	***	***
Position x Time	6	---	***	***
Position x Side	3	---	---	---
Side x Time	3	---	---	---
Position x Side x Time	9	---	---	---

***Denotes statistical significance for the effect at alpha = 0.001.
---Denotes no statistical significance ($P \geq 0.05$).

TABLE 3.2.21. TWO-SAMPLE CONTRASTS AND TRENDS FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC AND 24 HR

	Significance Times at 30, 60, and 120 Sec, and 24 Hr		
	Lengths	Widths	Areas
Trends			
Estimates Averaged Over Sides and Times for Linear Fit With Positions	---	---	---
Estimates Averaged Over Sides and Times for Quadratic Fit With Positions	*	***	***
Estimates Averaged Over Positions and Sides for Linear Fit With Times	***	***	***
Contrasts: Contralateral Difference Averaged ...			
At Position 1 Over All Times	---	---	---
At Position 2 Over All Times	---	---	---
At Position 3 Over All Times	---	---	---
At Position 4 Over All Times	---	---	---
Over All Positions and Times	---	---	---
For Linear Fit With Positions	---	---	---
For Quadratic Fit With Positions	---	---	---
At Time 30 Sec Over All Positions	---	---	---
At Time 60 Sec Over All Positions	---	---	---
At Time 120 Sec Over All Positions	---	---	---
At Time 24 Hr Over All Positions	---	---	---
For Linear Fit With Times	---	---	---
For Quadratic Fit With Times	---	---	---

*Denotes statistical significance for the trend at alpha = 0.05.
***Denotes statistical significance for the trend at alpha = 0.001.
---Denotes no statistical significance ($P \geq 0.05$).

TABLE 3.3.1. LESION LENGTHS (in mm) FOR 0.5 µl HD FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr
1	B4518	19	25	19	27	15	30	55	25
1	B4550	24	21	20	28	22	23	19	24
1	B4516	18	24	22	28	18	48	39	25
1	B4540	*	*	23	25	27	19	21	35
1	B4521	18	21	22	26	24	25	24	28
1	B4546	15	22	17	23	12	19	18	21
1	B4585	16	17	16	21	8	19	18	19
1	B4572	19	20	23	34	32	18	26	32
1	B4514	16	21	25	29	15	17	20	25
1	B4581	21	22	20	38	15	24	18	40
1	B4515	14	18	21	29	30	17	17	26
1	B4582	20	26	17	23	14	16	14	23
1	B4502	12	24	13	24	16	16	17	25
1	B4511	16	20	20	29	17	19	24	30
1	B4600	17	17	22	30	18	34	24	33
1	B4675	20	21	19	22	14	21	15	23
2	B4624	12	14	24	34	17	17	21	27
2	B4553	11	21	16	22	13	14	18	23
2	B4623	14	22	27	26	17	22	23	25
2	B4683	13	18	20	24	18	26	22	20

*Dosing error; lesion site not estimated.

TABLE 3.3.2. LESION WIDTHS (in mm) FOR 0.5 µl HD FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr
1	B4518	6	7	9	22	4	9	16	23
1	B4550	19	9	17	26	7	13	18	25
1	B4516	8	11	9	24	4	7	9	27
1	B4540	*	*	11	23	8	9	14	25
1	B4521	5	9	10	21	8	9	10	22
1	B4546	4	7	6	19	6	7	10	22
1	B4585	3	4	5	22	3	3	4	22
1	B4572	9	9	15	27	12	6	15	25
1	B4514	4	6	12	25	3	4	11	24
1	B4581	6	14	15	32	4	8	10	29
1	B4515	4	7	8	20	6	5	9	21
1	B4582	10	13	9	22	5	9	12	20
1	B4502	4	10	8	15	4	7	8	15
1	B4511	5	9	10	25	4	8	14	27
1	B4600	5	7	9	16	5	9	14	20
1	B4675	8	9	10	20	4	8	7	19
2	B4624	3	6	14	22	3	4	6	24
2	B4553	2	7	6	16	3	5	7	19
2	B4623	6	12	14	25	4	8	13	29
2	B4683	5	8	12	20	4	13	13	19

*Dosing error; lesion site not estimated.

TABLE 3.3.3. LESION AREAS (in mm^2) FOR 0.5 µl HD FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr
1	B4518	89	137	134	466	47	212	691	451
1	B4550	358	148	267	571	121	235	268	471
1	B4516	113	207	155	528	57	264	276	530
1	B4540	*	*	199	451	170	134	231	687
1	B4521	71	148	173	429	151	177	188	484
1	B4546	47	121	80	343	57	104	141	363
1	B4585	38	53	63	363	19	45	57	328
1	B4572	134	141	271	721	301	85	306	628
1	B4514	50	99	236	569	35	53	173	471
1	B4581	99	242	236	955	47	151	141	911
1	B4515	44	99	132	455	141	67	120	429
1	B4582	157	265	120	397	55	113	132	361
1	B4502	38	188	82	283	50	88	107	294
1	B4511	63	141	157	569	53	119	264	636
1	B4600	67	93	155	377	71	240	264	518
1	B4675	126	148	149	345	44	132	82	343
2	B4624	28	66	264	587	40	53	99	509
2	B4553	17	115	75	276	31	55	99	343
2	B4623	66	207	297	511	53	138	235	569
2	B4683	51	113	188	377	57	265	225	298

*Dosing error; lesion site not estimated.

TABLE 3.3.4. AVERAGE LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) USING M258A1 I AND II MATERIAL IN BULK VERSUS FIELD KIT PACKAGING IN THE RABBIT MODEL SCREEN FOR TEST CANDIDATES AGAINST 0.5 µl OF HD DECONTAMINATED AT 1.0, 3.0, AND 5.0 MIN AND 24 HR

	M258A1 I & II Bulk				M258A1 I & II Field Kit			
Response	1.0 Min	3.0 Min	5.0 Min	24 Hr	1.0 Min	3.0 Min	5.0 Min	24 Hr
Lengths	16.6	20.7	20.3	27.1	18.1	22.2	22.7	26.5
Widths	6.1	8.6	10.5	22.1	5.1	7.6	11.0	22.9
Areas	87.1	143.9	171.6	478.6	80.0	136.5	204.9	481.2

TABLE 3.3.5. LESION LENGTHS (in mm) FOR 0.5 µl L FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr
1	B5430	16	18	18	23	18	21	19	18
1	B5481	15	17	18	23	18	19	17	25
1	B5398	17	19	19	20	16	19	18	25
1	B5380	17	16	18	22	17	19	18	30
1	B5435	16	18	18	24	15	*	16	28
1	B5361	16	15	18	20	20	15	16	24
1	B5428	*	15	15	20	16	16	18	25
1	B5449	16	19	15	25	*	18	18	25
1	B5421	15	18	15	22	18	16	16	28
1	B5459	15	15	15	24	20	15	16	25
1	B5441	13	15	14	25	15	14	14	30
1	B5483	18	17	19	30	15	17	18	29
1	B5424	17	18	16	25	*	*	18	25
1	B5282	14	16	17	22	15	18	18	24
1	B5429	16	18	20	19	16	18	24	29
1	B5348	16	19	18	22	*	17	20	22
2	B5420	18	17	18	26	21	20	15	**
2	B5472	18	19	19	29	16	15	17	**
2	B5407	16	*	*	28	15	*	*	**
2	B5487	16	21	17	34	17	17	18	**

*Dosing error; lesion site not estimated.
**Dosing error; dose was not applied to this site.

TABLE 3.3.6. LESION WIDTHS (in mm) FOR 0.5 µl L FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr
1	B5430	5	5	7	15	4	5	6	6
1	B5481	5	6	5	19	5	6	5	16
1	B5398	6	6	8	16	7	7	6	17
1	B5380	4	5	4	15	4	5	5	15
1	B5435	6	6	7	16	6	*	7	17
1	B5361	5	6	5	13	4	6	6	14
1	B5428	*	7	8	14	6	8	8	13
1	B5449	6	6	8	18	*	8	7	19
1	B5421	7	7	7	16	5	6	9	14
1	B5459	6	7	7	17	5	8	8	24
1	B5441	5	6	5	19	4	7	6	17
1	B5483	6	7	7	20	7	8	9	17
1	B5424	5	7	8	17	*	*	8	18
1	B5282	5	6	5	18	5	5	6	17
1	B5429	4	6	6	15	5	6	6	14
1	B5348	6	7	6	15	*	7	8	12
2	B5420	5	7	6	17	7	8	8	**
2	B5472	6	6	6	20	5	6	6	**
2	B5407	5	*	*	22	5	*	*	**
2	B5487	5	6	6	19	4	7	10	**

*Dosing error; lesion site not estimated.
**Dosing error; dose was not applied to this site.

TABLE 3.3.7. LESION AREAS (in mm^2) FOR 0.5 µl L FOLLOWED BY M258A1 I AND II DECONTAMINATION FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC AND 24 HR

Replicate	Animal Number	M258A1 I & II Bulk				M258A1 I & II Field Kit			
		30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr
1	B5430	63	71	99	271	57	82	89	85
1	B5481	59	80	71	343	71	89	67	314
1	B5398	80	89	119	251	88	104	85	334
1	B5380	53	63	57	259	53	75	71	353
1	B5435	75	85	99	301	71	*	88	374
1	B5361	63	71	71	204	63	71	75	264
1	B5428	*	82	94	220	75	100	113	255
1	B5449	75	89	94	353	*	113	99	373
1	B5421	82	99	82	276	71	75	113	308
1	B5459	71	82	82	320	79	94	101	471
1	B5441	51	71	55	373	47	77	66	400
1	B5483	85	93	104	471	82	107	127	387
1	B5424	67	99	100	334	*	*	113	353
1	B5282	55	75	67	311	59	71	85	320
1	B5429	50	85	94	224	63	85	113	319
1	B5348	75	104	85	259	*	93	126	207
2	B5420	71	93	85	347	115	126	94	**
2	B5472	85	89	89	455	63	71	80	**
2	B5407	63	*	*	484	59	*	*	**
2	B5487	63	99	80	507	53	93	141	**

*Dosing error; lesion site not estimated.
**Dosing error; dose was not applied to this site.

TABLE 3.3.8. AVERAGE LESION LENGTHS (in mm), WIDTHS (in mm), AND AREAS (in mm^2) USING M258A1 I AND II MATERIAL IN BULK VERSUS FIELD KIT PACKAGING IN THE RABBIT MODEL SCREEN FOR TEST CANDIDATES AGAINST 0.5 µl OF L DECONTAMINATED AT 30, 60, AND 120 SEC AND 24 HR

	M258A1 I & II Bulk				M258A1 I & II Field Kit			
Response	30 Sec	60 Sec	120 Sec	24 Hr	30 Sec	60 Sec	120 Sec	24 Hr
Lengths	16.1	17.4	17.2	22.9	16.9	17.3	17.6	25.8*
Widths	5.4	6.3	6.4	16.4	5.2	6.6	7.1*	15.0
Areas	67.7	85.3	85.7	298.2	68.7	89.8	97.1	307.5

*Significantly greater ($P < 0.05$, one-sided) than mean lesion size at corresponding exposure period for bulk M258A1 I and II decontamination.

(Note: Four animals were not dosed with L at the site receiving decontamination on the field kit side at 24 hr. The averages shown at that time are based on 16 animals only).

TABLE 3.4.1. LESION LENGTHS (in mm) FOR 0.5 µl OF HD FOLLOWED BY M258A1 I AND II DECONTAMINATION AT EITHER 20, 45, AND 75 SEC OR AT 10, 15, AND 60 SEC (LEFT SIDE) AND DECONTAMINATION WITH A 5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR (BOTH SIDES)

Position	1		2		3	
Side	Left	Right	Left	Right	Left	Right
			August 7, 1985			
Animal			Time to Decontamination			
Number	20 Sec	24 Hr	45 Sec	24 Hr	75 Sec	24 Hr
B4689	10	20	14	22	13	17
B4764	12	23	15	27	16	24
B4718	12	22	12	21	12	22
B4778	20	24	16	25	32	22
B4717	14	20	13	19	14	19
B4779	12	21	15	21	16	22
B4709	12	21	16	20	16	22
B4777	14	19	15	18	12	19
			August 9, 1985			
Animal			Time to Decontamination			
Number	10 Sec	24 Hr	15 Sec	24 Hr	60 Sec	24 Hr
B4695	12	22	18	23	19	26
B4745	10	29	10	32	14	30
B4728	13	20	19	21	15	22
B4746	14	19	28	20	31	21
B4734	14	35	10	34	14	39
B4738	10	22	9	23	12	23
B4733	10	19	12	20	12	20
B4770	9	22	12	25	13	23

TABLE 3.4.2. LESION LENGTH RATIOS (LEFT/RIGHT) FOR 0.5 µl OF HD FOLLOWED BY M258A1 I AND II DECONTAMINATION AT EITHER 20, 45, AND 75 SEC OR AT 10, 15, AND 60 SEC (LEFT SIDE) AND DECONTAMINATION WITH A 5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR (BOTH SIDES)

Animal Number	August 7, 1985 Time to Decontamination		
	20 Sec	45 Sec	75 Sec
B4689	0.500	0.636	0.765
B4764	0.522	0.556	0.667
B4718	0.545	0.571	0.545
B4778	0.833	0.640	1.455*
B4717	0.700	0.684	0.737
B4779	0.571	0.714	0.727
B4709	0.571	0.800	0.727
B4777	0.737	0.833	0.632

Animal Number	August 9, 1985 Time to Decontamination		
	10 Sec	15 Sec	60 Sec
B4695	0.545	0.783	0.731
B4745	0.345	0.313	0.467
B4728	0.650	0.905	0.682
B4746	0.737	1.400*	1.476*
B4734	0.400	0.294	0.359
B4738	0.455	0.391	0.522
B4733	0.52[illegible]	0.600	0.600
B4770	0.409	0.480	0.565

*Outlier at alpha = 0.05, two-sided.

TABLE 3.4.3. CALCULATION OF TERM TO SUBTRACT FROM LESION LENGTH ESTIMATE (in mm) AT POSITION n FOR COMPARISON WITH ESTIMATE AT 24-HR CONTROL SITE IN MREF PROTOCOL 22 VALIDATION DATA

Validation at 1.25, 5.0, and 10 Min to Decontamination

Position	Mean*	Time to Decontamination	Correction Term**
1	18.7	1.25 Min	0.2
2	19.2	5.0 Min	0.7
3	19.2	10.0 Min	0.7
4	18.5	24 Hr	-

Validation at 1.0, 3.0, and 5.0 Min to Decontamination

Position	Mean***	Time to Decontamination	Correction Term**
1	21.0	1.0 Min	0.4
2	22.3	3.0 Min	1.7
3	21.9	5.0 Min	1.3
4	20.6	24 Hr	-

*The mean of all lesion lengths for the position indicated in Tables 3.2.1 through 3.2.4.
**Calculated as the mean at position n less the mean at position 4.
***The mean of all lesion lengths for the position indicated in Tables 3.2.5 through 3.2.8.
-Not applicable.

TABLE 3.4.4. HD LESION SIZE LENGTHS (in mm) FROM MREF PROTOCOL 22 VALIDATION WORK; DECONTAMINATION WITH M258A1 I AND II AT 1.25, 5.0, AND 10.0 MIN FOLLOWED BY DECONTAMINATION AT 24 HR WITH 5 PERCENT SODIUM HYPOCHLORITE SOLUTION

Animal Number	Time to Decontamination			
	1.25 Min	5.0 Min	10.0 Min	24 Hr
B1592	24	24	24	24
B1346	22	24	24	24
B1304	14	20	24	25
B1342	24	20	25	30
B1634	20	25	25	28
B1336	20	16	19	22
B1508	24	30	37	30
B1483	30	28	34	25
B1459	20	22	22	24
B1325	36	35	35	42
B1306	28	34	35	35
B1338	15	25	25	25
B1529	22	35	30	30
B1326	25	30	30	28
B1496	25	34	30	35
B1349	24	28	35	22
B1609	18	20	24	26
B1731	30	27	31	37
B1507	25	35	35	35
B1732	25	37	46	38
B1309	25	38	40	45
B1698	25	37	35	35
B1307	22	32	34	35
B1655	20	25	23	35

TABLE 3.4.5. HD LESION SIZE LENGTHS (in mm) FROM MREF PROTOCOL 22 VALIDATION WORK; DECONTAMINATION WITH M258A1 I AND II AT 1.0, 3.0, AND 5.0 MIN FOLLOWED BY DECONTAMINATION AT 24 HR WITH 5 PERCENT SODIUM HYPOCHLORITE SOLUTION

Animal Number	Time to Decontamination			
	1.0 Min	3.0 Min	5.0 Min	24 Hr
B3886	18	19	24	25
B3938	17	*	24	25
B4003	16	21	26	30
B3946	18	16	19	22
B3911	16	21	19	23
B3959	*	14	17	20
B3924	18	20	21	27
B4059	19	20	22	20
B3892	*	21	22	24
B3981	18	22	18	25
B3887	29	22	28	29
B4043	17	20	24	26
B3930	18	21	19	27
B3939	22	23	27	38
B3920	17	23	21	26
B3968	16	29	22	34
B4213	17	23	22	37
B4139	31	24	22	31
B4119	*	24	24	34
B3954	25	22	19	25
B4103	19	24	23	30
B4142	19	28	31	42
B4120	21	23	22	39
B3957	28	42	29	28

*Data not used due to error in dosing.

TABLE 3.4.6. RATIO OF LESION LENGTH ESTIMATES* AT 75, 300, OR 600 SEC TO DECONTAMINATION WITH M258A1 I AND II RELATIVE TO LESION LENGTH ESTIMATE AT 24 HR TO DECONTAMINATION WITH 5 PERCENT SODIUM HYPOCHLORITE SOLUTION

Animal Number	Time to Decontamination		
	75 Sec	300 Sec	600 Sec
B1592	0.992	0.971	0.971
B1346	0.908	0.971	0.971
B1304	0.552	0.772	0.932
B1342	0.793	0.643	0.810
B1634	0.707	0.868	0.868
B1336	0.900	0.695	0.832
B1508	0.793	0.977	1.210
B1483	1.192**	1.092	1.332
B1459	0.825	0.888	0.888
B1325	0.852	0.817	0.817
B1306	0.794	0.951	0.980
B1338	0.592	0.972	0.972
B1529	0.727	1.143	0.977
B1326	0.886	1.046	1.046
B1496	0.709	0.951	0.837
B1349	1.082**	1.241**	1.559**
B1609	0.685	0.742	0.896
B1731	0.805	0.711	0.819
B1507	0.709	0.980	0.980
B1732	0.653	0.955	1.192
B1309	0.551	0.829	0.873
B1698	0.709	1.037	0.980
B1307	0.623	0.894	0.951
B1655	0.566	0.894	0.637

*Corrected for positional effects.
**Outlier at alpha = 0.05, two-sided.

TABLE 3.4.7. RATIO OF LESION LENGTH ESTIMATES* AT 60, 180, OR 300 SEC TO DECONTAMINATION WITH M258A1 I AND II RELATIVE TO LESION LENGTH ESTIMATE AT 24 HR TO DECONTAMINATION WITH 5 PERCENT SODIUM HYPOCHLORITE SOLUTION

Animal Number	Time to Decontamination		
	60 Sec	180 Sec	300 Sec
B3886	0.704	0.692	0.908
B3938	0.664	**	0.908
B4003	0.520	0.643	0.823
B3946	0.800	0.650	0.805
B3911	0.678	0.839	0.770
B3959	**	0.615	0.785
B3924	0.652	0.678	0.730
B4059	0.930	0.915	1.035
B3892	**	0.804	0.863
B3981	0.704	0.812	0.668
B3887	0.986	0.700	0.921
B4043	0.638	0.704	0.873
B3930	0.652	0.715	0.656
B3939	0.568	0.561	0.676
B3920	0.638	0.819	0.758
B3968	0.459	0.803	0.609
B4213	0.449	0.576	0.559
B4139	0.987	0.719	0.668
B4119	**	0.656	0.668
B3954	0.984	0.812	0.708
B4103	0.620	0.743	0.723
B4142	0.443	0.626	0.707
B4120	0.528	0.546	0.531
B3957	0.986	1.439***	0.989

*Corrected for positional effects.
**Data not used due to error in dosing.
***Outlier at alpha = 0.05, two-sided.

TABLE 3.4.8. DATA SET FOR NONLINEAR REGRESSION ON LESION GROWTH RATIOS WHERE GROWTH = LESION LENGTH LESS 10 mm

MREF Protocol 22 Validation Data (Adjusted for Position Effects)

Corrected Length Growth/Non-Decontaminated Control Lesion Length Growth					
75 Sec	300 Sec	600 Sec	60 Sec	180 Sec	300 Sec
0.986	0.950	0.950	0.507	0.487	0.847
0.843	0.950	0.950	0.440	*	0.847
0.253	0.620	0.887	0.280	0.465	0.735
0.690	0.465	0.715	0.633	0.358	0.642
0.544	0.794	0.794	0.431	0.715	0.592
0.817	0.442	0.692	*	0.230	0.570
0.690	0.965	1.315	0.447	0.488	0.571
1.320**	1.153	1.553	0.860	0.830	1.070
0.700	0.807	0.807	*	0.664	0.764
0.806	0.759	0.759	0.507	0.687	0.447
0.712	0.932	0.972	0.979	0.542	0.879
0.320	0.953	0.953	0.413	0.519	0.794
0.590	1.215	0.965	0.447	0.547	0.453
0.822	1.072	1.072	0.414	0.404	0.561
0.592	0.932	0.772	0.413	0.706	0.606
1.150**	1.440**	2.030**	0.233	0.721	0.446
0.488	0.581	0.831	0.244	0.419	0.396
0.733	0.604	0.752	0.981	0.586	0.510
0.592	0.972	0.972	*	0.513	0.529
0.529	0.939	1.261	0.973	0.687	0.513
0.423	0.780	0.837	0.430	0.615	0.585
0.592	1.052	0.972	0.269	0.509	0.616
0.472	0.852	0.932	0.366	0.390	0.369
0.392	0.572	0.492	0.978	1.680**	0.983

MREF Protocol 23 Optimization D Data

Lesion Length Growth/Contralateral Control Growth					
20 Sec	45 Sec	75 Sec	10 Sec	15 Sec	60 Sec
0.000	0.333	0.429	0.167	0.615	0.563
0.154	0.294	0.429	0.000	0.000	0.200
0.167	0.182	0.167	0.300	0.818	0.417
0.714	0.400	1.450**	0.440	1.400**	1.480**
0.400	0.333	0.444	0.160	0.000	0.138
0.182	0.455	0.500	0.000	-0.077	0.154
0.182	0.600	0.500	0.000	0.200	0.200
0.444	0.625	0.222	-0.083	0.133	0.231

*Data not used due to error in dosing.
**Outlier at alpha = 0.05, two-sided.

TABLE 3.4.9. CALCULATION OF TIME TO DECONTAMINATION FOR SPECIFIED LESION GROWTH RATIOS (R_G)

$$R_G = 1-B_2e^{(-B_1t)}$$

or

$$t = \frac{\ln\left(\frac{1-R_G}{B_2}\right)}{-B_1}$$

where

$B_1 = 0.003561$
$B_2 = 0.8113$

Lesion Growth Ratio	Time (Sec)	Selected Times to Decontamination
0.00	-59*	
0.05	-44*	
0.10	-29*	
0.15	-13*	
0.20	4	
0.25	22	(1/2 min)
0.30	41	
0.35	62	
0.40	85	
0.45	109	
0.50	136	(2-1/2 min)
0.55	166	
0.60	199	
0.65	236	
0.70	279	
0.75	331	(5-1/2 min)
0.80	393	
0.85	474	
0.90	588	
0.95	783	

*Negative time value indicates that the lesion growth ratio was not attainable.

TABLE 3.5.1. DRAIZE IRRITATION SCORES FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER M258A1 I AND II OR DISTILLED WATER AT 0.75, 1.5, AND 3.5 MIN*

Animal	M258A1 I, II						Control		Distilled Water					
	0.75 Min		1.5 Min		3.5 Min		24 Hr		0.75 Min		1.5 Min		3.5 Min	
Number	R	E	R	E	R	E	R	E	R	E	R	E	R	E
Date of Studies: 8/21/86														
C2047M	2	2	2	2	2	2	2	4	2	3	2	3	2	3
C2070F	2	2	2	2	1	2	3	3	3	4	3	4	3	4
C2022M	2	2	2	2	2	2	3	3	2	4	2	4	2	4
C2085F	2	3	2	3	2	3	3	4	3	4	3	4	3	4
C2020M	2	2	2	2	2	3	3	4	3	4	3	4	3	4
C2059F	2	2	2	2	2	2	3	4	3	4	3	4	3	4
C2005M	2	2	2	2	2	2	3	4	3	4	3	4	3	4
C2100F	1	2	1	1	1	1	2	3	2	3	2	3	2	3
C2006M	2	2	2	1	2	1	2	2	2	4	2	4	2	4
C2071F	2	2	2	2	2	1	3	3	3	4	3	4	3	4
C2010M	2	3	2	2	2	2	3	4	3	4	3	4	3	4
C2073F	2	2	2	2	2	1	3	4	3	4	3	4	3	4
Date of Studies: 8/26/86														
C2017M	2	2	2	2	2	2	3	4	3	4	3	4	3	4
C2078F	1	1	1	1	2	2	4	3	4	3	4	3	4	3
C2015M	1	1	1	1	1	1	2	1	4	3	4	3	4	3
C2057F	3	3	3	3	3	3	3	2	4	4	4	4	4	4
C2002M	1	2	1	2	1	2	3	4	4	4	4	4	4	4
C2097F	2	2	2	2	2	2	3	3	4	4	4	4	4	4
C2041M	2	1	2	1	3	2	3	3	4	3	4	3	4	3
C2065F	1	1	1	1	2	2	2	3	4	3	4	3	4	3
C2014M	1	2	1	2	1	2	3	3	3	4	3	4	3	4
C2077F	1	2	1	2	1	2	4	4	4	4	4	4	4	4
C2007M	1	1	1	1	2	2	4	3	4	4	4	4	4	4
C2085F	1	1	1	1	1	2	4	4	4	4	4	4	4	4

*Followed by decontamination with 0.5 percent sodium hypochlorite solution and three distilled water rinses at 4 hr after dosing.
R = Erythema
E = Edema

TABLE 3.5.2. PERCENT REFLECTION SCORES FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER M258A1 I AND II OR DISTILLED WATER AT 0.75, 1.5, and 3.5 MIN*

Animal Number	M258A1 I, II 0.75 Min	M258A1 I, II 1.5 Min	M258A1 I, II 3.5 Min	Control 24 Hr	Distilled Water 0.75 Min	Distilled Water 1.5 Min	Distilled Water 3.5 Min
			Date of Studies: 8/21/86				
C2047M	57.0	59.8	56.3	55.3	51.5	51.1	56.6
C2070F	89.8	91.1	86.4	80.4	83.2	81.3	80.0
C2022M	82.1	86.2	86.0	83.8	80.8	82.8	79.4
C2085F	81.6	80.0	78.1	79.4	77.4	80.3	79.0
C2020M	86.3	85.2	79.9	91.4	87.5	88.9	88.5
C2059F	76.2	76.8	73.6	76.7	74.2	74.8	77.6
C2005M	87.2	86.4	83.6	84.6	79.2	81.4	84.1
C2100F	88.6	89.3	84.9	88.1	90.9	88.3	89.6
C2006M	**	82.1	85.2	90.5	84.0	82.1	85.9
C2071F	91.1	87.4	85.0	84.5	88.3	84.9	85.6
C2010M	86.0	88.9	85.6	84.5	82.3	81.9	82.8
C2073F	91.4	81.6	88.3	85.4	85.4	87.9	86.0
			Date of Studies: 8/26/86				
C2017M	74.6	80.6	79.5	85.1	77.6	80.4	79.1
C2078F	89.3	91.0	87.8	78.3	85.8	87.7	82.5
C2015M	86.4	87.9	83.0	85.5	83.7	84.6	84.8
C2057F	76.8	87.5	85.3	80.6	84.1	82.9	84.1
C2002M	95.3	96.8	98.6	93.6	90.1	87.6	92.1
C2097F	92.9	93.3	95.2	98.8	85.5	93.8	91.0
C2041M	82.9	95.0	95.5	91.0	81.8	80.6	88.1
C2065F	96.0	92.8	83.5	87.8	85.1	90.1	87.4
C2014M	97.7	88.5	87.6	88.4	89.8	88.6	90.8
C2077F	85.7	74.5	87.7	77.1	74.6	77.4	77.3
C2007M	94.1	99.2	87.8	98.7	87.1	92.3	98.8
C2085F	93.6	86.9	83.8	77.8	78.9	79.2	84.6

*Followed by decontamination with 0.5 percent sodium hypochlorite solution and three distilled water rinses at 4 hr after dosing.
**Data not collected.

TABLE 3.5.3. MEAN DRAIZE ERYTHEMA AND EDEMA SCORES AND MEAN REFLECTANCE SCORES FOR 0.5 µl OF HD FOLLOWED BY DECONTAMINATION WITH EITHER M258A1 I AND II OR DISTILLED WATER AT 0.75, 1.5, AND 3.5 MIN*

	M258A1 I, II			Control	Distilled Water		
	0.75 Min	1.5 Min	3.5 Min	24 Hr	0.75 Min	1.5 Min	3.5 Min
Erythema	1.7	1.7	1.8	3.0	3.3**	3.3**	3.3**
Edema	1.9	1.8	1.9	3.3	3.8**	3.8**	3.8**
Total Draize Score***	3.5	3.4	3.7	6.3	7.0**	7.0**	7.0**
Reflectance (color filter not used)	86.2	86.2	84.5	84.5	82.0**	83.0**	84.0

*Followed by decontamination with 0.5 percent sodium hypochlorite solution and three distilled water rinses at 4 hr after dosing.
**Significantly ($P < 0.05$) more irritated than the contralateral M258A1 I and II standard at the same time.
***The mean total Draize score does not always equal the sum of the mean erythema and mean edema scores due to rounding.

TABLE 3.5.4. BINOMIAL IRRITATION RESPONSE SCORES** FOR RH1-86 VERSUS M258A1 I AND II STANDARD IN THE MREF PROTOCOL 22 SCREEN

Observer	Day	Time to Decontamination 1 Min	3 Min	5 Min	Total	Screen Results
KA	1	1/8	1/7	0/5		
LG	2	3/8	2/6	3/7		
LG	3	0/4	2/7	2/7		
	Sum	4/20++	5/20++	5/19++	14/59	Passed++
TK	1	1/7	2/7	1/6		
TK	2	3/8	3/7	3/7		
TK	3	1/7	2/7	2/6		
	Sum	5/22++	7/21+	6/19+	18/62	Passed++
LW	1	1/4	1/5	0/4		
BD	2	5/8	5/6	3/6		
BD	3	1/7	2/7	1/5		
	Sum	7/19+	8/18+	4/15+	19/52	Passed+

**The binomial irritation score is the number of candidate-decontaminated HD dose sites more irritated than the contralateral standard decontaminated dose sites divided by the number of qualifying (nontied) lesion pairs.

++Candidate-decontaminated dose sites showed significantly ($P < 0.05$) less HD irritation than standard-decontaminated dose sites.

+Equivalent ($P > 0.05$) HD irritation for candidate and M258A1 I, II standard.

TABLE 3.5.5. BINOMIAL IRRITATION RESPONSE SCORES** FOR RH4-86 VERSUS M258A1 I AND II STANDARD IN THE MREF PROTOCOL 22 SCREEN

Observer	Day	Times to Decontamination 1 Min	3 Min	5 Min	Total	Screen Results
KA	1	0/4	4/6	1/4		
KA	2	0/5	2/7	1/6		
KA	3	3/8	0/7	1/7		
	Sum	3/17++	6/20+	3/17++	12/54	Passed++
CK	1	2/5	5/8	1/6		
BD	2	0/5	2/7	1/6		
BD	3	3/8	0/6	3/7		
	Sum	5/18+	7/21+	5/19++	17/58	Passed++
LA	1	2/7	5/7	1/5		
TK	2	2/6	2/6	1/7		
TK	3	2/7	0/6	2/8		
	Sum	6/20+	7/19+	4/20++	17/59	Passed++

**The binomial irritation score is the number of candidate-decontaminated HD dose sites more irritated than the contralateral standard-decontaminated dose sites divided by the number of qualifying (nontied) lesion pairs.

++Candidate-decontaminated dose sites showed significantly ($P < 0.05$) less HD irritation than standard-decontaminated dose sites.

+Equivalent ($P > 0.05$) HD irritation for candidate and M258A1 I, II standard.

TABLE 3.5.6. BINOMIAL IRRITATION RESPONSE SCORES** FOR RH5-86 VERSUS M258A1 I AND II STANDARD IN THE MREF PROTOCOL 22 SCREEN

Observer	Day	Times to Decontamination 1 Min	3 Min	5 Min	Total	Screen Results
KA	1	2/4	7/7	4/7		
KA	2	3/7	2/7	1/7		
KA	3	4/8	3/6	1/4		
	Sum	9/19+	12/20+	6/18+	27/57	Passed+
CK	1	4/6	8/8	4/6		
BD	2	3/8	1/8	3/8		
BD	3	4/8	4/7	0/3		
	Sum	11/22+	13/23+	7/17+	31/62	Passed+
LA	1	2/5	8/8	4/6		
TK	2	3/8	1/8	3/8		
TK	3	4/8	5/8	1/5		
	Sum	9/21+	14/24+	8/19+	31/64	Passed+

**The binomial irritation score is the number of candidate-decontaminated HD dose sites more irritated than the contralateral standard-decontaminated dose sites divided by the number of qualifying (nontied) lesion pairs.

+Equivalent ($P > 0.05$) HD irritation for candidate and M258A1 I, II standard.

TABLE 3.5.7. BINOMIAL IRRITATION RESPONSE SCORES** FOR RH6-86 VERSUS M258A1 I AND II STANDARD IN THE MREF PROTOCOL 22 SCREEN

Observer	Day	Times to Decontamination 1 Min	3 Min	5 Min	Total	Screen Results
KA	1	3/6	4/7	4/5		
LG	2	7/8	6/7	5/8		
LG	3	6/7	5/6	5/6		
	Sum	16/21-	15/20-	14/19-	45/60	Failed-
TK	1	4/8	3/6	4/6		
TK	2	7/8	7/7	6/8		
TK	3	5/6	5/6	5/5		
	Sum	16/22-	15/19-	15/19-	46/60	Failed-
LW	1	3/5	4/4	5/5		
BD	2	7/8	8/8	7/8		
BD	3	5/6	6/6	4/5		
	Sum	15/19-	18/18-	16/18-	49/55	Failed-

**The binomial irritation score is the number of candidate-decontaminated HD dose sites more irritated than the contralateral standard-decontaminated dose sites divided by the number of qualifying (nontied) lesion pairs.

-Candidate-decontaminated dose sites showed significantly ($P < 0.05$) more HD irritation than standard-decontaminated dose sites.

TABLE 3.5.8. COMPARISON OF RESULTS BETWEEN BINOMIAL IRRITATION RESPONSE AND LESION LENGTH ANALYSES FOR FOUR ROHM AND HAAS CANDIDATE DECONTAMINANT SYSTEMS VERSUS THE M258A1 I AND II STANDARD

Times to Decontamination:	Lesion Lengths 1 Min	3 Min	5 Min		Binomial Irritation Scores 1 Min	3 Min	5 Min
Candidate System				Observer			
RH1-86	++	++	++	TK	++	+	+
RH4-86	++	++	++	KA	++	+	++
RH5-86	++	++	++	KA	+	+	+
RH6-86	+	+	+	TK	-	-	-
				Screen Results			
RH1-86		Passed++				Passed++	
RH4-86		Passed++				Passed++	
RH5-86		Passed++				Passed+	
RH6-86		Passed+				Failed-	

-The candidate decontamination system was significantly ($P < 0.05$) not as effective as the M258A1 I, II standard system against HD.
++The candidate decontamination system was significantly ($P < 0.05$) more effective than the M258A1 I, II standard system against HD.
+The candidate decontamination system was not significantly ($P < 0.05$) different from the M258A1 I, II standard system against HD.

TABLE 3.5.9. IMAGE LESION LENGTHS (in mm) OBTAINED BY MANUAL PLANIMETRY ON PROJECTED PHOTOGRAPHS OF RABBIT BACKS

Animal Number	M258A1 I & II				Distilled Water		
	0.75 Min	1.5 Min	3.5 Min	Control	0.75 Min	1.5 Min	3.5 Min
C2047M	19.1	16.6	14.2	20.7	25.5	22.7	29.4
C2070F	9.6	15.4	15.4	17.7	33.6	33.1	33.4
C2022M	18.3	12.2	14.8	20.7	49.1	43.9	38.8
C2085F	12.6	14.2	14.3	24.5	29.6	37.8	31.5
C2020M	21.9	14.2	18.7	22.1	41.2	38.0	30.4
C2059F	13.6	14.0	17.8	23.3	41.2	26.8	27.2
C2005M	11.2	12.2	17.0	21.1	40.5	40.1	33.9
C2100F	12.3	11.0	11.7	23.2	23.1	28.3	24.4
C2006M	*	9.0	11.9	27.0	31.3	41.0	44.1
C2071F	9.9	12.6	14.4	22.8	*	33.2	41.4
C2010M	19.8	12.4	25.8	26.7	31.8	30.3	28.5
C2073F	10.6	14.3	15.3	26.3	25.7	26.9	33.2
C2017M	11.9	11.5	13.1	18.8	25.9	24.9	22.4
C2078F	11.8	10.0	14.0	16.5	31.7	29.9	30.9
C2015M	10.8	10.6	11.1	16.4	22.9	19.9	23.0
C2057F	13.6	*	16.8	23.3	47.3	37.1	34.5
C2002M	12.4	12.8	10.3	19.5	27.4	32.4	32.8
C2097F	8.7	10.6	16.5	20.9	47.9	45.8	41.9
C2041M	12.4	14.6	14.2	17.7	27.4	29.9	30.4
C2065F	*	*	*	19.8	29.4	21.3	31.4
C2014M	*	13.6	13.3	17.4	29.1	26.5	29.1
C2077F	10.5	13.9	13.9	25.8	27.4	24.8	25.4
C2007M	8.9	8.6	17.0	20.0	20.1	19.6	22.3
C2085F	15.0	18.5	17.8	31.0	26.3	35.0	25.0

*Data not used due to error in dosing HD.

TABLE 3.5.10. IMAGE LESION WIDTHS (in mm) OBTAINED BY MANUAL PLANIMETRY ON PROJECTED PHOTOGRAPHS OF RABBIT BACKS

Animal Number	M258A1 I & II				Distilled Water		
	0.75 Min	1.5 Min	3.5 Min	Control	0.75 Min	1.5 Min	3.5 Min
C2047M	6.1	7.8	6.8	12.4	9.9	9.6	13.8
C2070F	3.1	4.2	5.5	12.6	8.0	12.4	13.4
C2022M	6.5	5.8	10.3	18.5	12.7	11.0	17.6
C2085F	6.4	5.0	9.4	13.9	11.5	14.7	15.8
C2020M	9.3	6.2	11.0	17.2	10.9	13.3	21.7
C2059F	5.5	6.0	9.8	18.1	9.7	9.0	10.2
C2005M	3.9	5.7	5.7	24.1	7.8	9.4	15.5
C2100F	7.4	6.5	6.8	21.6	8.0	14.0	17.6
C2006M	*	4.9	7.6	24.4	8.5	10.1	15.8
C2071F	3.9	6.1	8.1	18.0	*	8.6	11.2
C201 'M	9.5	7.1	9.2	25.9	12.3	14.9	21.9
C207 ₃F	6.1	8.7	7.2	18.1	12.2	12.1	14.1
C2017M	4.2	4.4	6.9	9.9	11.2	13.2	15.7
C2078F	3.6	3.0	4.6	7.9	11.3	11.7	12.5
C2015M	3.2	3.2	3.0	8.5	11.2	11.8	10.6
C2057F	3.0	*	6.4	15.1	11.2	12.0	13.8
C2002M	3.4	4.0	4.1	13.1	8.4	10.4	11.4
C2097F	4.6	3.6	5.9	13.4	11.3	12.3	12.9
C2041M	3.6	3.3	4.2	13.4	7.3	10.6	9.3
C2065F	*	*	*	12.9	8.8	10.0	8.8
C2014M	*	3.3	5.3	11.9	10.7	12.6	12.9
C2077F	2.4	3.4	4.5	8.2	11.7	10.6	9.3
C2007M	1.5	2.5	4.3	6.5	3.3	5.0	7.0
C2085F	4.2	4.8	8.2	13.8	13.0	13.3	9.0

*Data not used due to error in dosing HD.

TABLE 3.5.11. LESION LENGTHS (in mm) OBTAINED BY RULER ON LIVE RABBIT BACKS

Animal Number	M258A1 I & II			Control	Distilled Water		
	0.75 Min	1.5 Min	3.5 Min		0.75 Min	1.5 Min	3.5 Min
C2047M	19	22	18	29	21	23	28
C2070F	11	16	18	21	35	34	27
C2022M	26	10	17	26	52	42	38
C2085F	14	16	18	28	29	32	26
C2020M	20	15	17	26	43	51	45
C2059F	13	14	17	26	35	32	32
C2005M	19	15	21	22	38	36	26
C2100F	13	13	14	21	21	26	22
C2006M	*	15	16	22	26	35	25
C2071F	15	15	16	25	*	31	27
C2010M	18	15	25	30	33	30	29
C2073F	14	15	17	35	24	29	30
C2017M	18	15	18	29	26	27	25
C2078F	20	12	25	28	33	34	29
C2015M	15	17	15	26	30	29	30
C2057F	25	*	29	26	45	37	33
C2002M	16	18	17	28	31	36	30
C2097F	13	15	20	30	38	37	31
C2041M	13	17	20	28	29	34	31
C2065F	*	*	*	27	39	37	30
C2014M	*	16	19	29	32	30	28
C2077F	12	15	17	29	34	30	37
C2007M	12	15	16	32	42	40	36
C2085F	12	15	17	30	27	27	32

*Data not used due to error in dosing HD.

TABLE 3.5.12. LESION WIDTHS (in mm) OBTAINED BY RULER ON LIVE RABBIT BACKS

Animal Number	M258A1 I & II			Control	Distilled Water		
	0.75 Min	1.5 Min	3.5 Min		0.75 Min	1.5 Min	3.5 Min
C2047M	9	8	8	15	11	10	12
C2070F	5	5	9	14	15	20	16
C2022M	7	5	9	17	16	16	19
C2085F	5	5	10	20	18	19	16
C2020M	12	6	8	12	15	15	17
C2059F	5	10	9	20	13	15	14
C2005M	5	7	8	16	14	13	11
C2100F	5	8	9	18	12	11	13
C2006M	*	4	7	18	14	10	17
C2071F	4	5	8	19	*	18	17
C2010M	6	5	9	17	15	15	21
C2073F	5	8	8	23	17	15	17
C2017M	8	7	11	18	14	17	17
C2078F	5	5	9	15	12	14	15
C2015M	6	7	7	14	17	15	16
C2057F	6	*	12	25	17	19	18
C2002M	4	5	7	17	14	17	15
C2097F	5	4	8	17	16	18	13
C2041M	4	6	12	20	16	16	18
C2065F	*	*	*	16	15	18	17
C2014M	*	5	10	17	17	19	19
C2077F	5	5	7	19	15	17	16
C2007M	4	5	9	18	14	15	16
C2085F	3	6	10	17	13	15	16

*Data not used due to error in dosing HD.

TABLE 3.5.13. MEAN LENGTHS AND WIDTHS (in mm) OBTAINED BY RULER OR MANUAL PLANIMETRY FROM LESIONS CAUSED BY 0.5 µl of HD FOLLOWED BY DECONTAMINATION WITH EITHER M258A1 I AND II OR DISTILLED WATER AT 0.75, 1.5, AND 3.5 MIN*

	M258A1 I & II				Distilled Water		
	0.75 Min	1.5 Min	3.5 Min	Control	0.75 Min	1.5 Min	3.5 Min
				Lengths			
Ruler	16.1	15.3	18.6	27.2	33.2	33.3	30.3
Planimetry	13.1**	12.9**	15.2**	21.8**	32.0	31.2	31.1
				Widths			
Ruler	5.6	6.0	8.9	17.6	14.8	15.7	16.1
Planimetry	4.8	5.0**	6.7**	15.0	10.0**	11.4**	13.4**

*Followed by decontamination with 0.5 percent sodium hypochlorite solution and three distilled water rinses at 4 hr after dosing.
**Measurement was significantly ($P < 0.05$, two-sided) underestimated by manual planimetry relative to ruler measurement.

APPENDIX D

Figures

FIGURE 3.1.1. MEAN LESION LENGTHS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

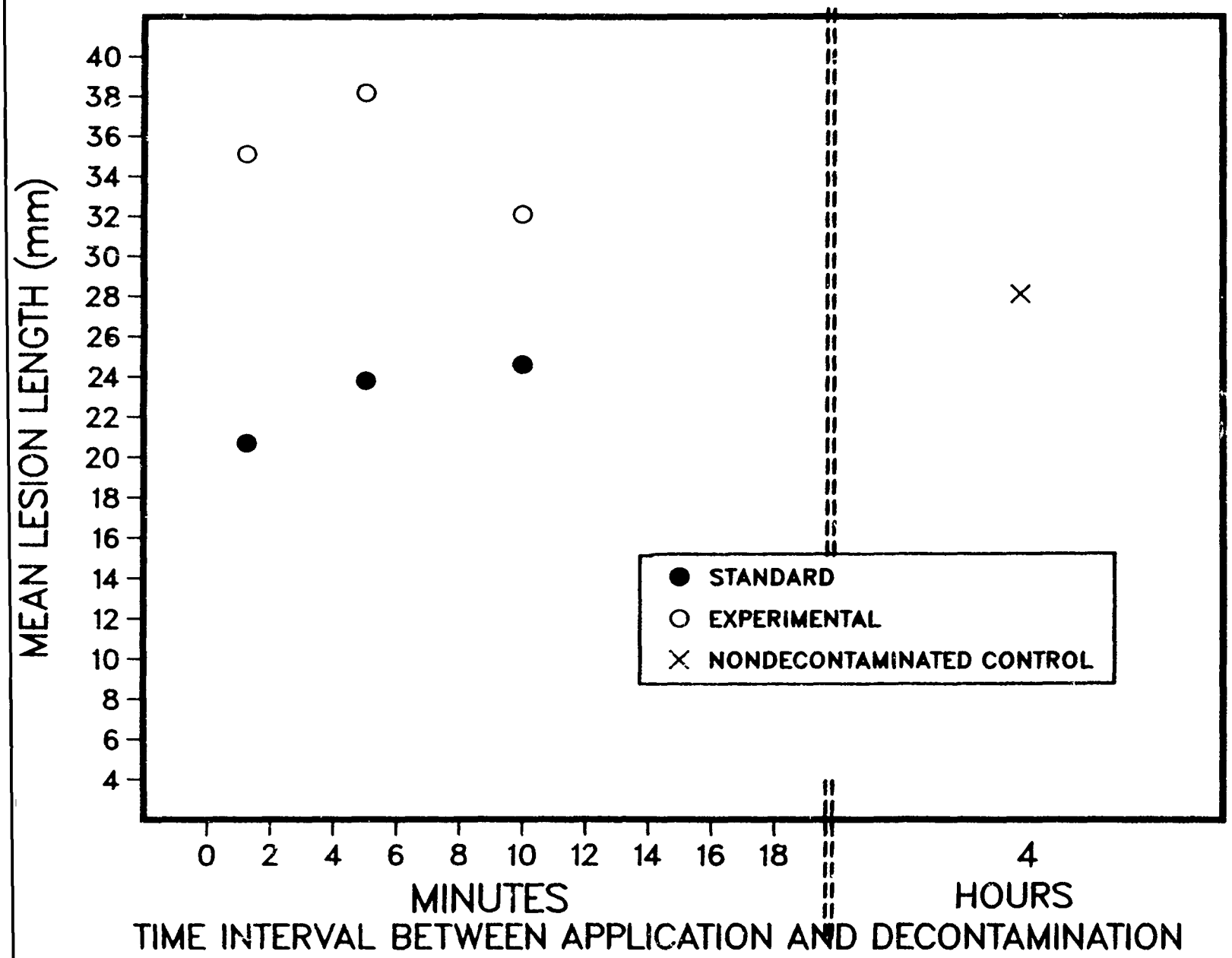


FIGURE 3.1.2. MEAN LESION WIDTHS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

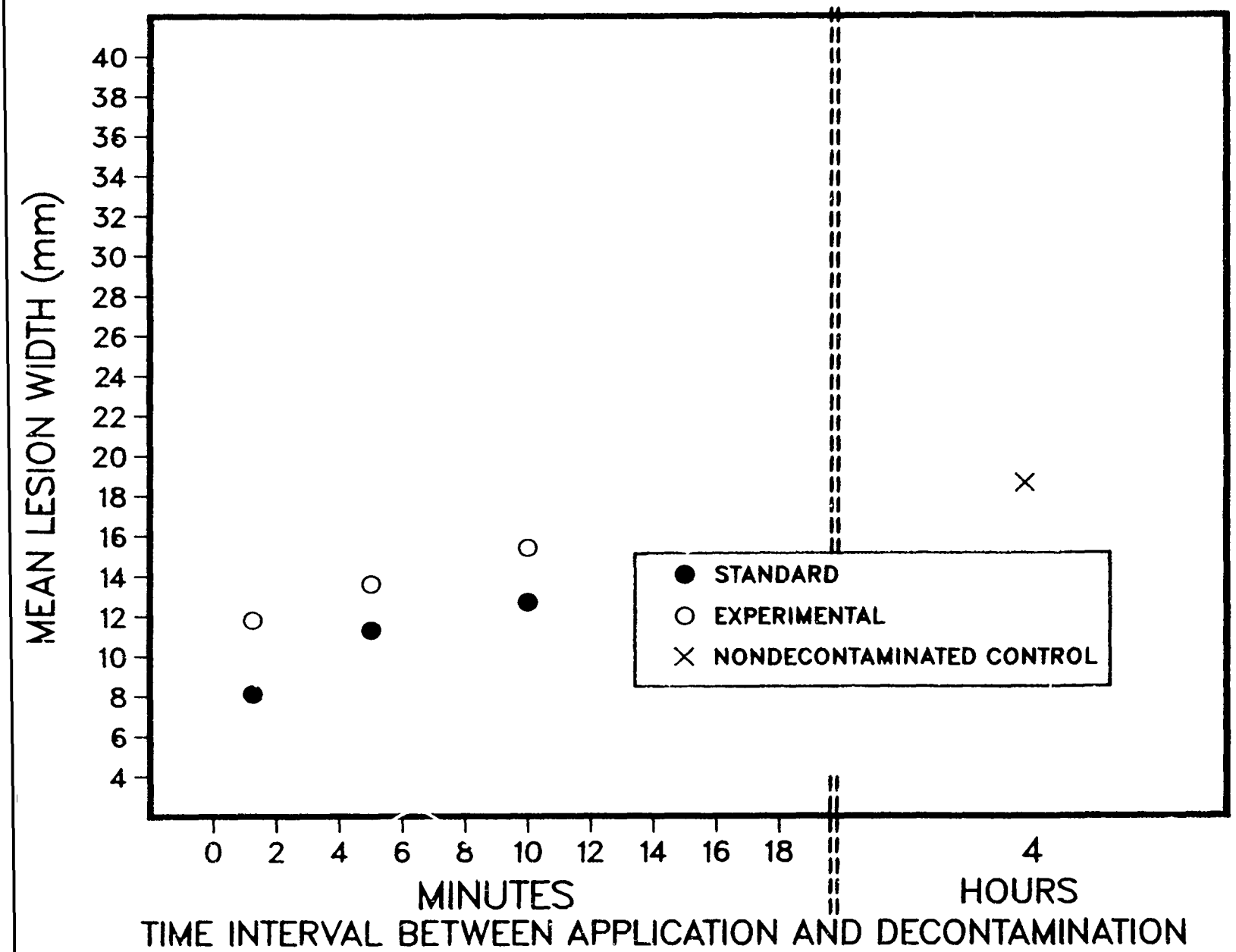


FIGURE 3.1.3. MEAN LESION AREAS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

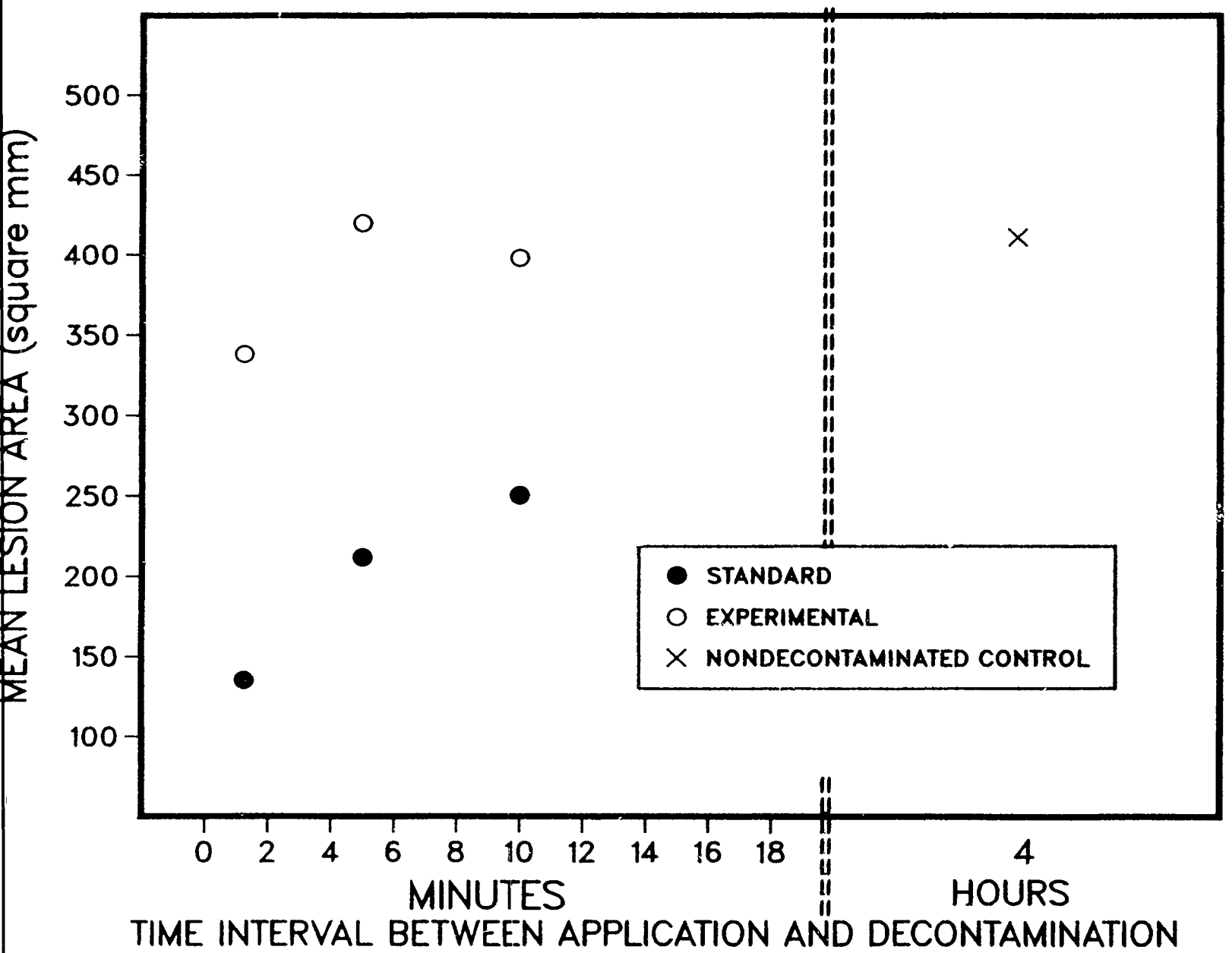


FIGURE 3.1.4. MEAN LESION LENGTHS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING

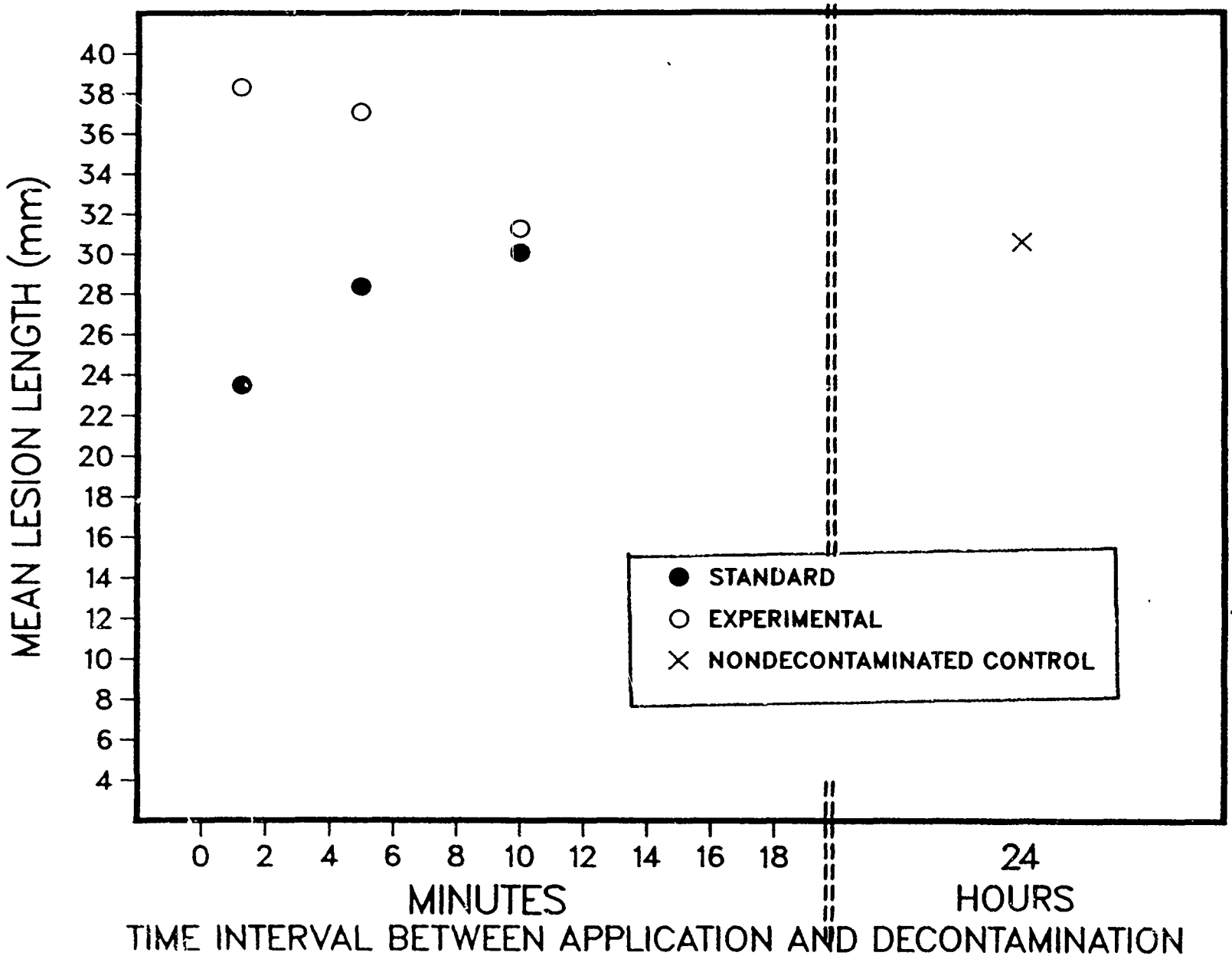


FIGURE 3.1.5. MEAN LESION WIDTHS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING

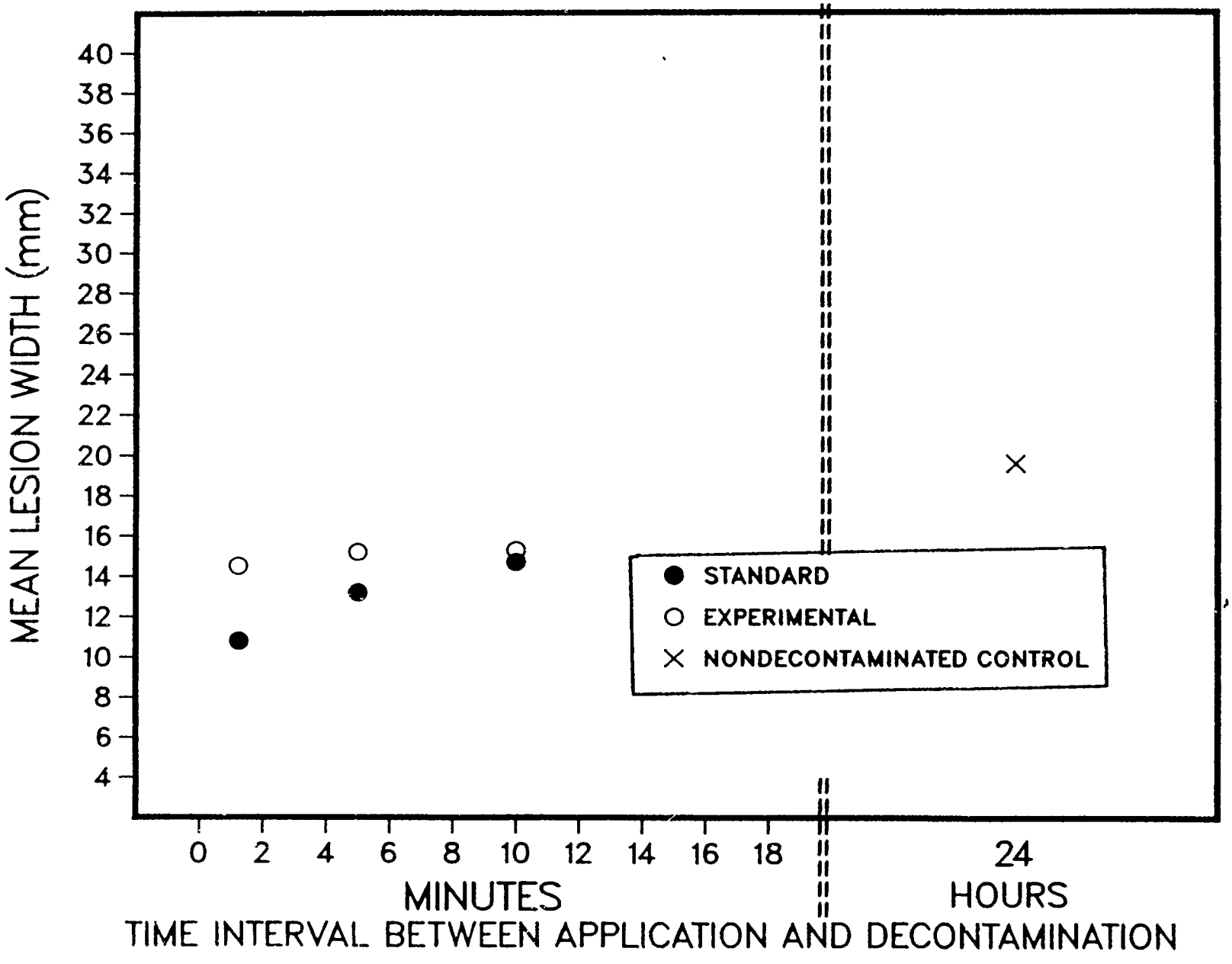


FIGURE 3.1.6. MEAN LESION AREAS FOR 0.5 µl OF HD DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING

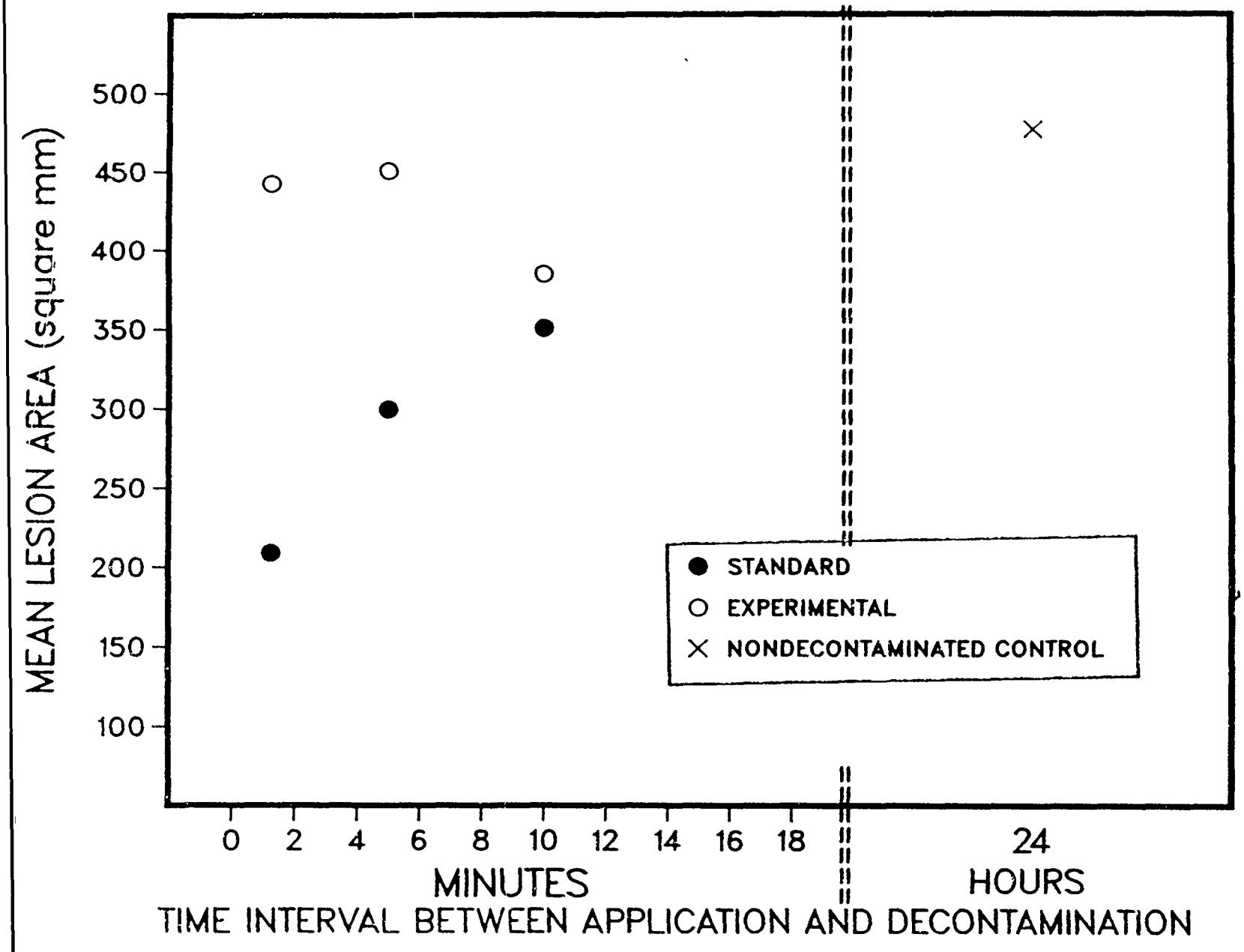


FIGURE 3.1.7. MEAN LESION LENGTHS FOR 0.5 µl OF L DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

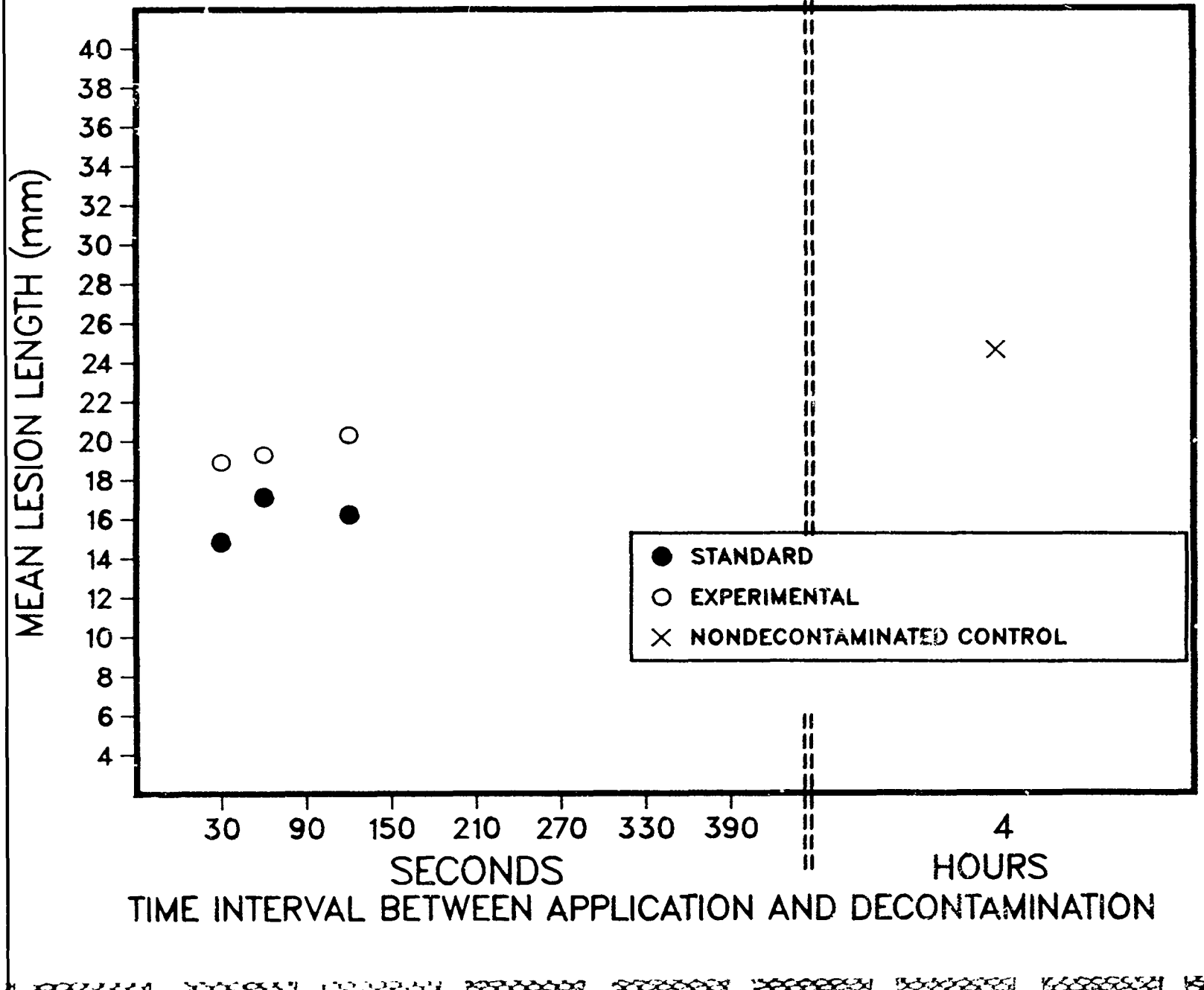


FIGURE 3.1.8. MEAN LESION WIDTHS FOR 0.5 µl OF L DECONTAMINATION WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

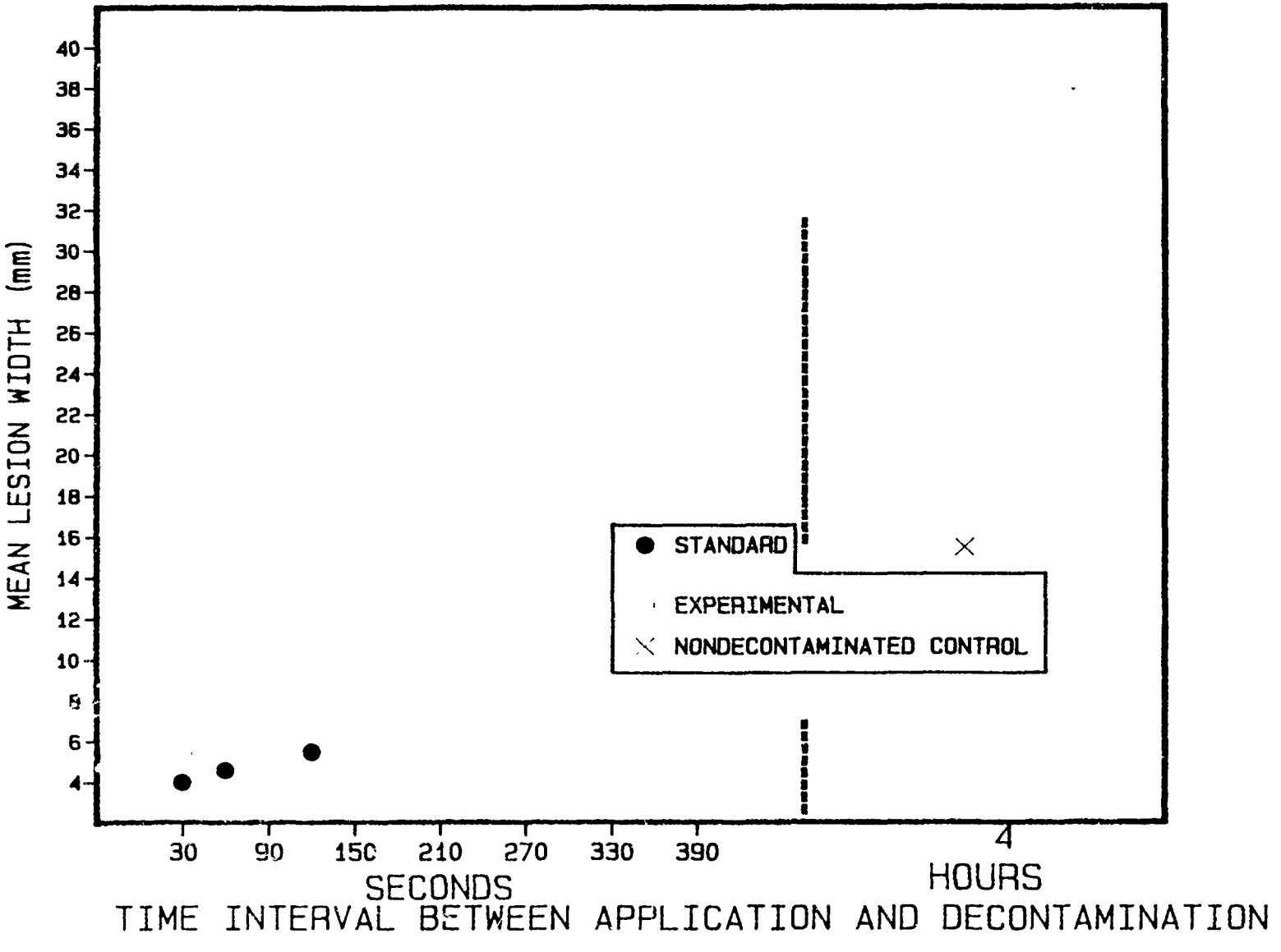


FIGURE 3.1.9. MEAN LESION AREAS FOR 0.5 µl OF L DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 4 HR AFTER DOSING

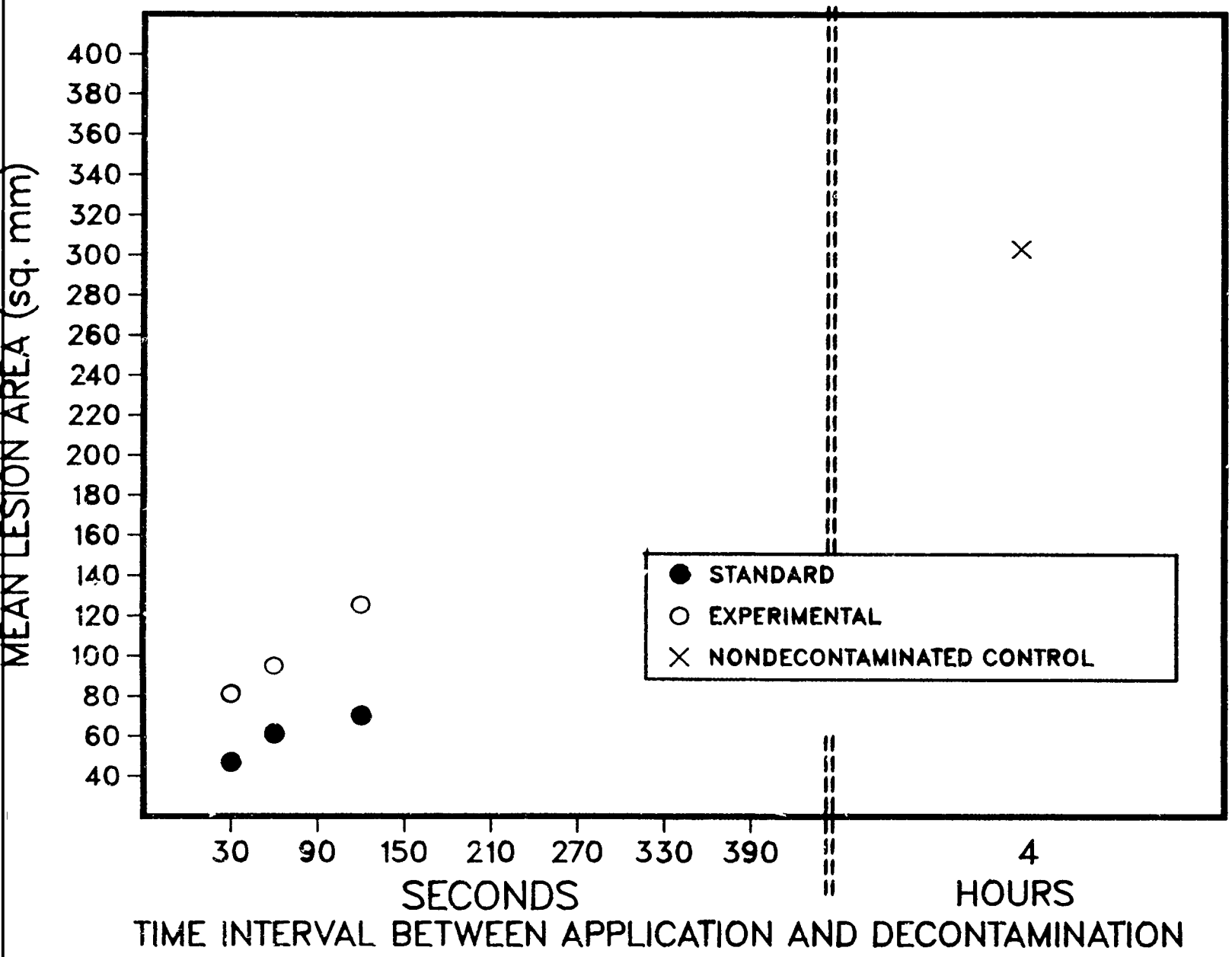
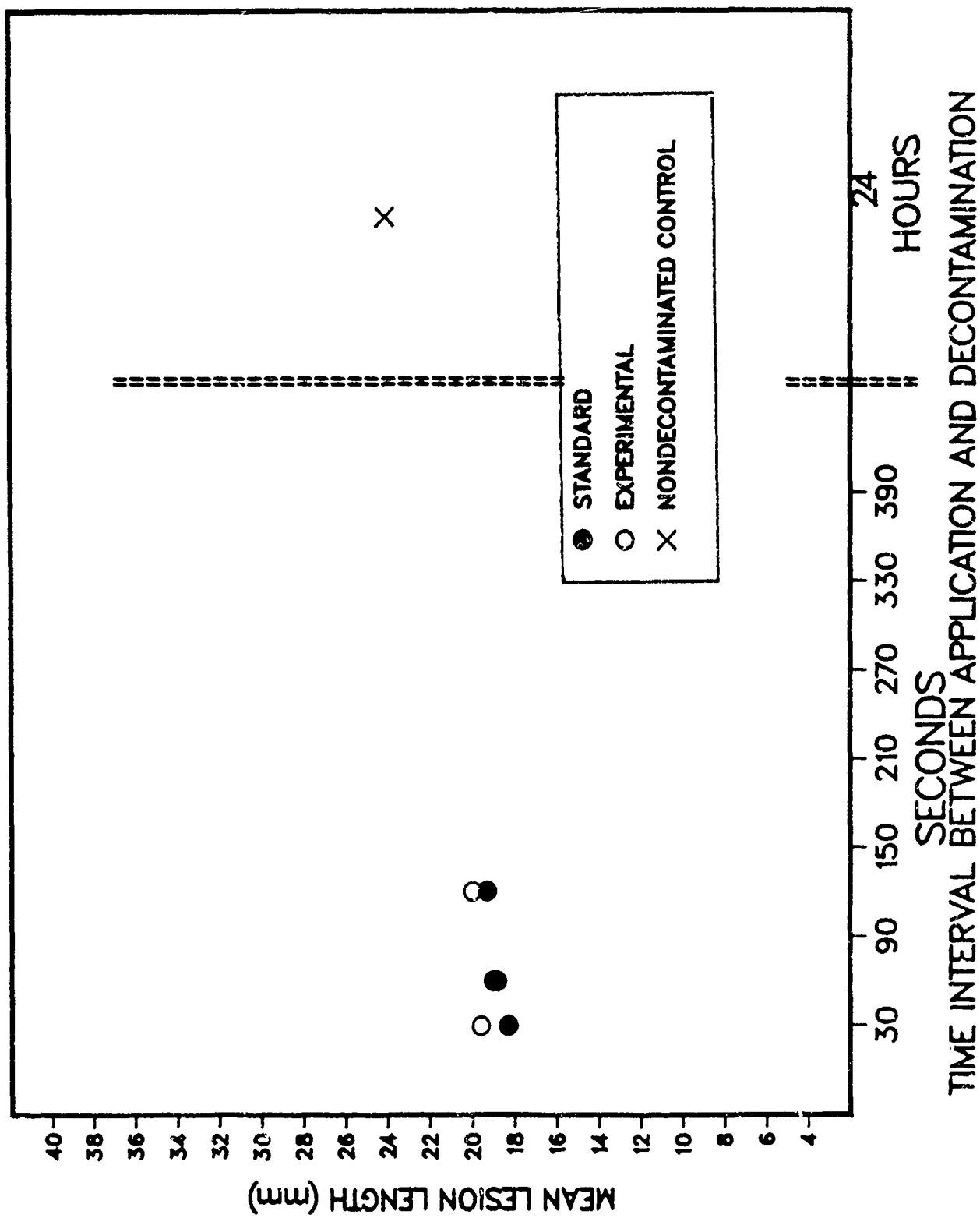


FIGURE 3.1.10. MEAN LESION LENGTHS FOR 0.5 µl OF L DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING



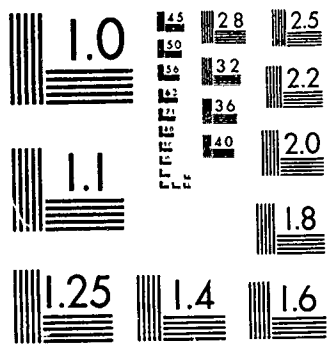

1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3

FIGURE 3.1.11. MEAN LESION WIDTHS FOR 0.5 µl OF L DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING

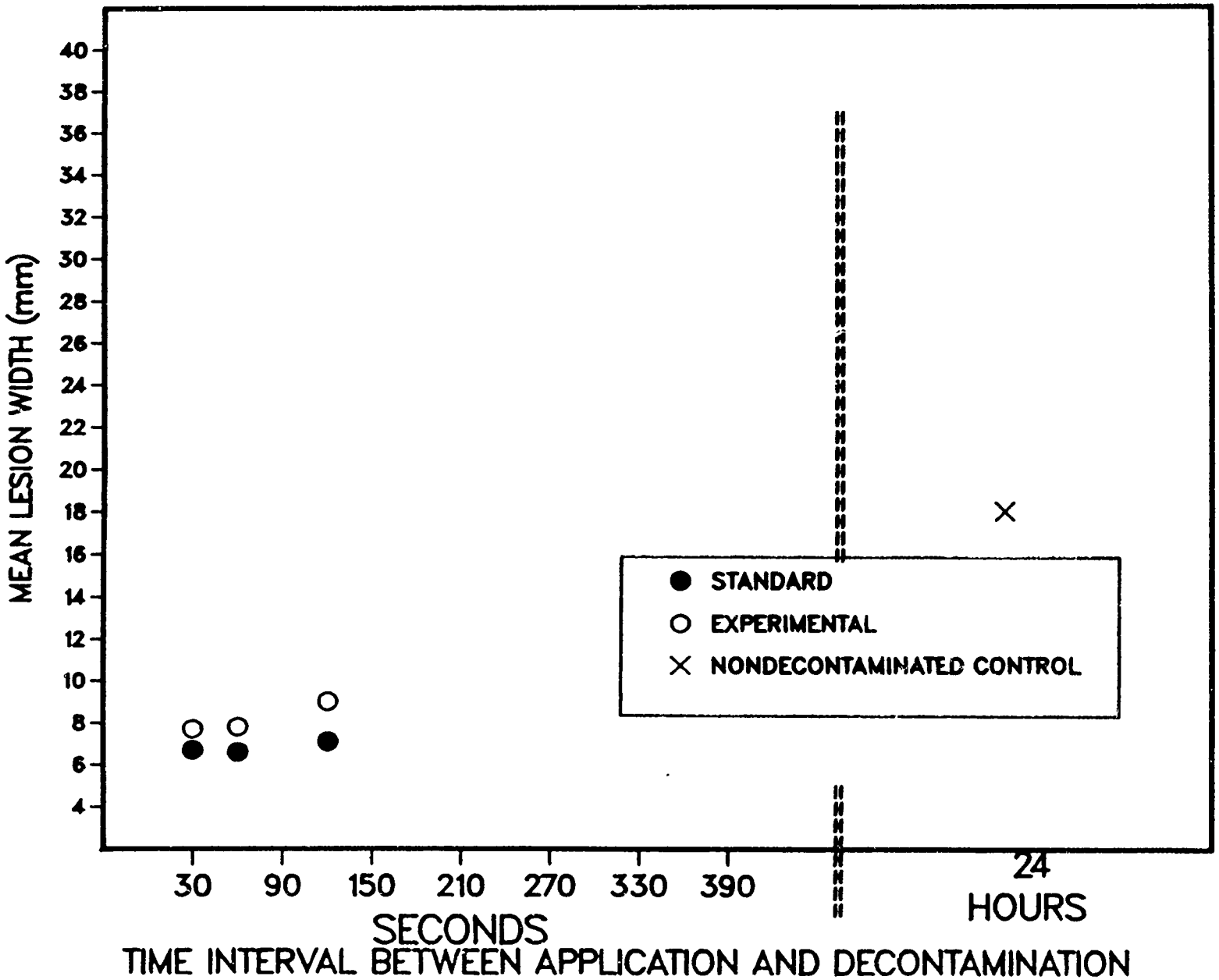


FIGURE 3.1.12. MEAN LESION AREAS FOR 0.5 µl OF L DECONTAMINATED WITH EITHER STANDARD M258A1 I AND II OR DISTILLED WATER DECONTAMINATION SYSTEM AND 5.0 PERCENT SODIUM HYPOCHLORITE SOLUTION AT 24 HR AFTER DOSING

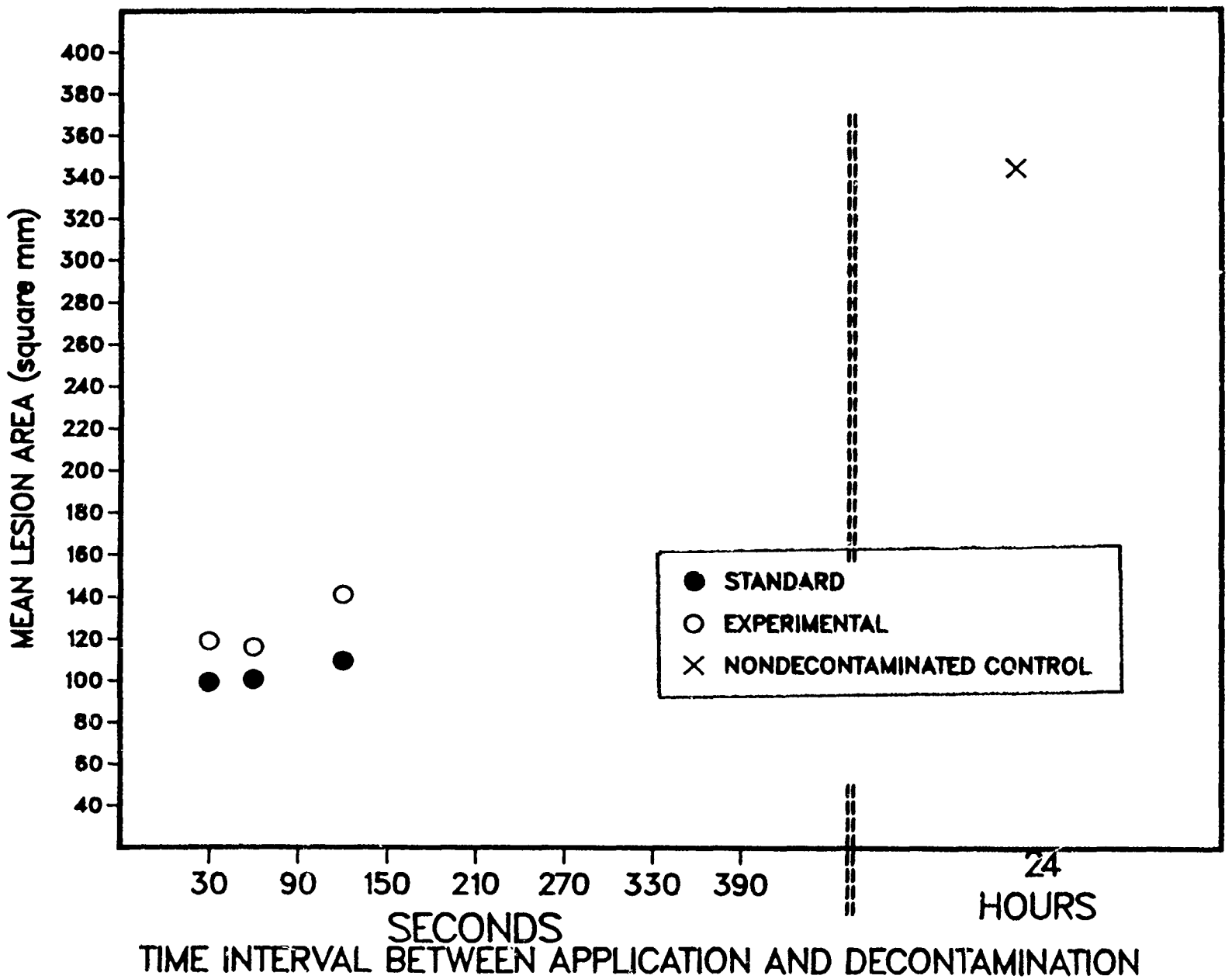


FIGURE 3.2.1. MEAN LESION LENGTHS FOR 0.5 µl of HD DECONTAMINATED WITH M258A1 I AND II AT 1.25, 5.0, AND 10.0 MIN, AND 24 HR AFTER DOSING

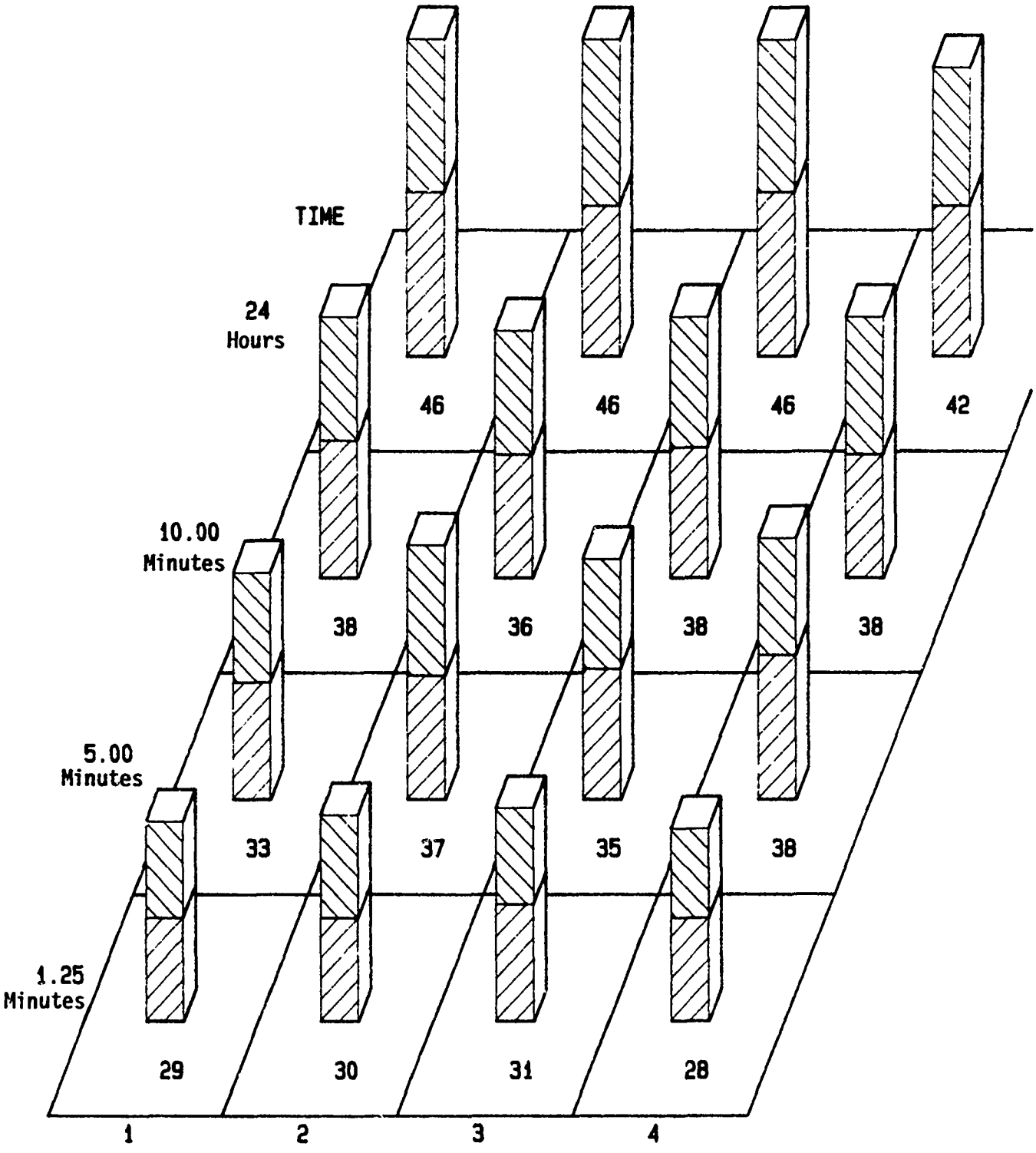


FIGURE 3.2.2. MEAN LESION WIDTHS FOR 0.5 µl of HD DECONTAMINATED WITH M258A1 I AND II AT 1.25, 5.0, and 10.0 MIN, AND 24 HR AFTER DOSING

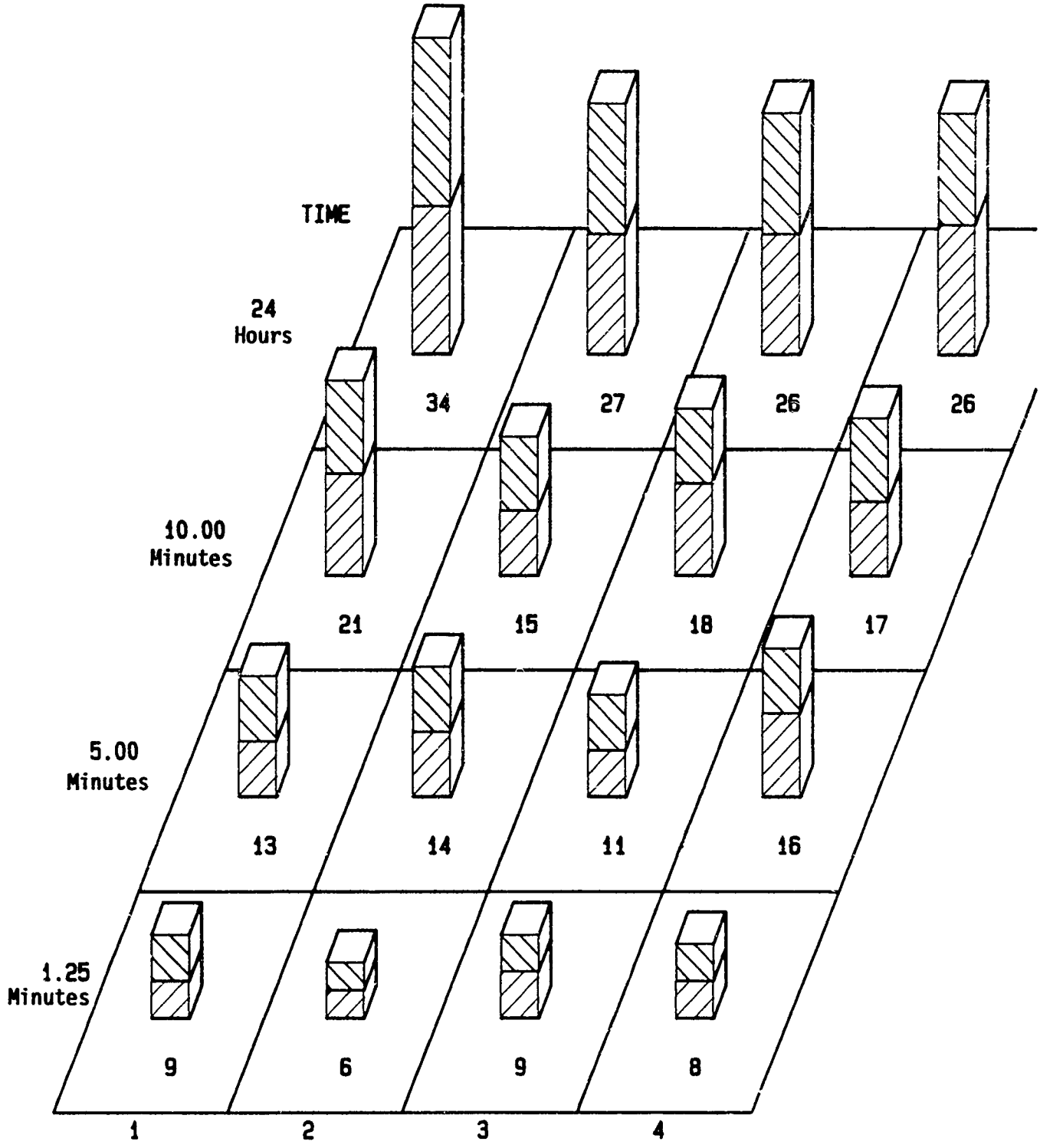


FIGURE 3.2.3. MEAN LESION AREAS FOR 0.5 µl of HD DECONTAMINATED WITH M258A1 I AND II AT 1.25, 5.0, and 10.0 MIN, AND 24 HR AFTER DOSING

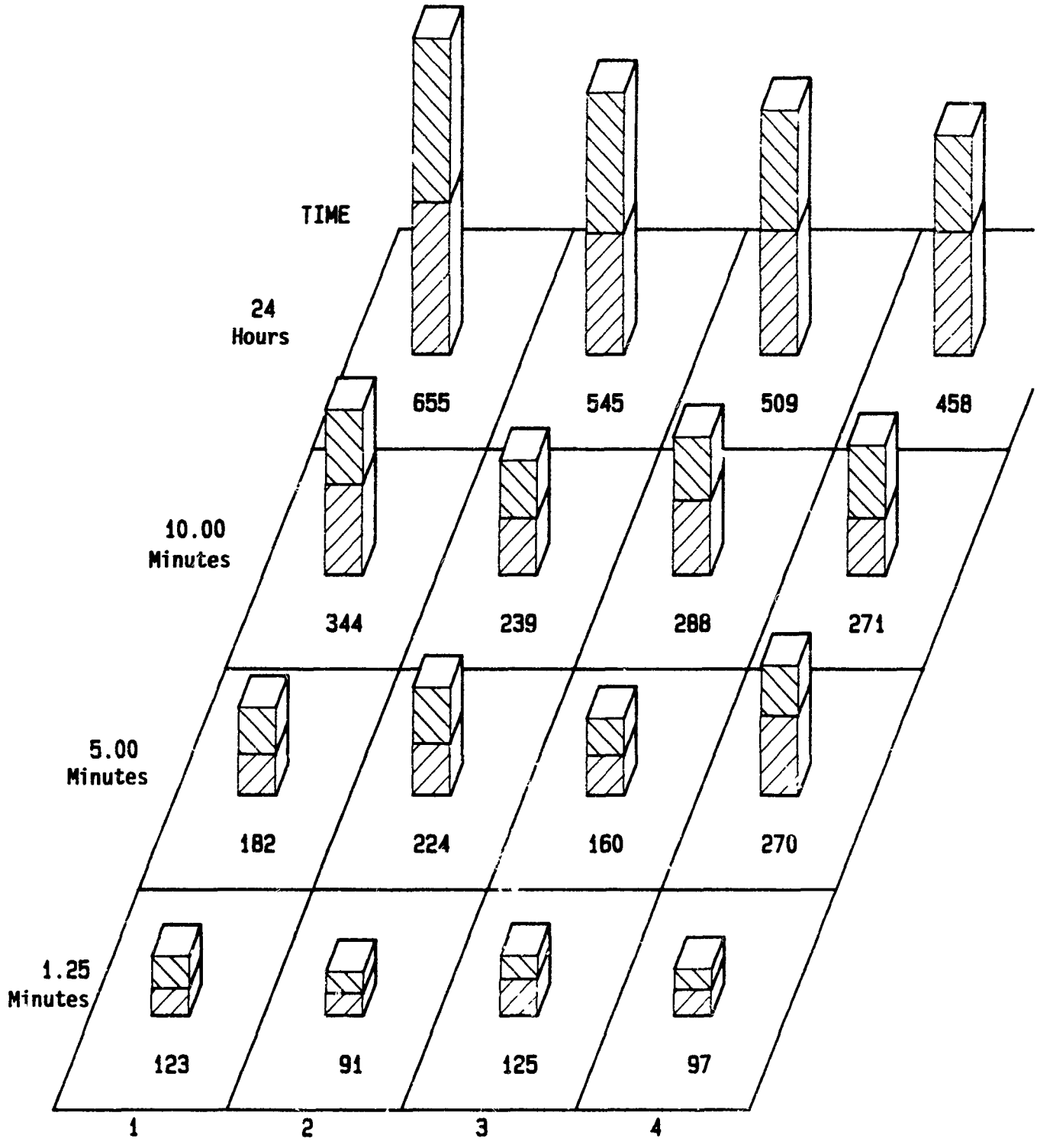


FIGURE 3.2.4. MEAN LESION LENGTHS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT 1.0, 3.0, and 5.0 MIN, AND 24 HR AFTER DOSING

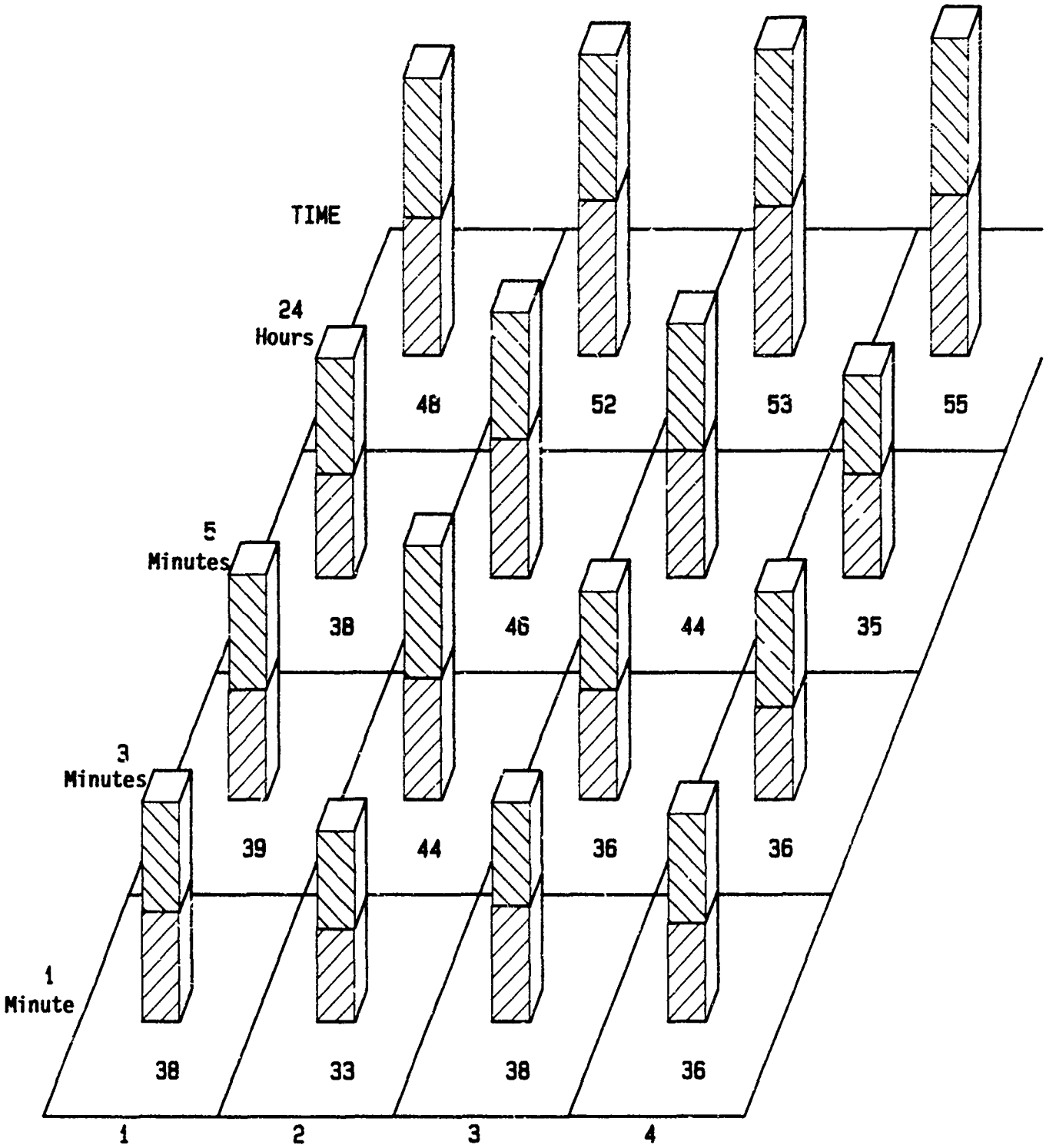


FIGURE 3.2.5. MEAN LESION WIDTHS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT 1.0, 3.0, and 5.0 MIN, AND 24 HR AFTER DOSING

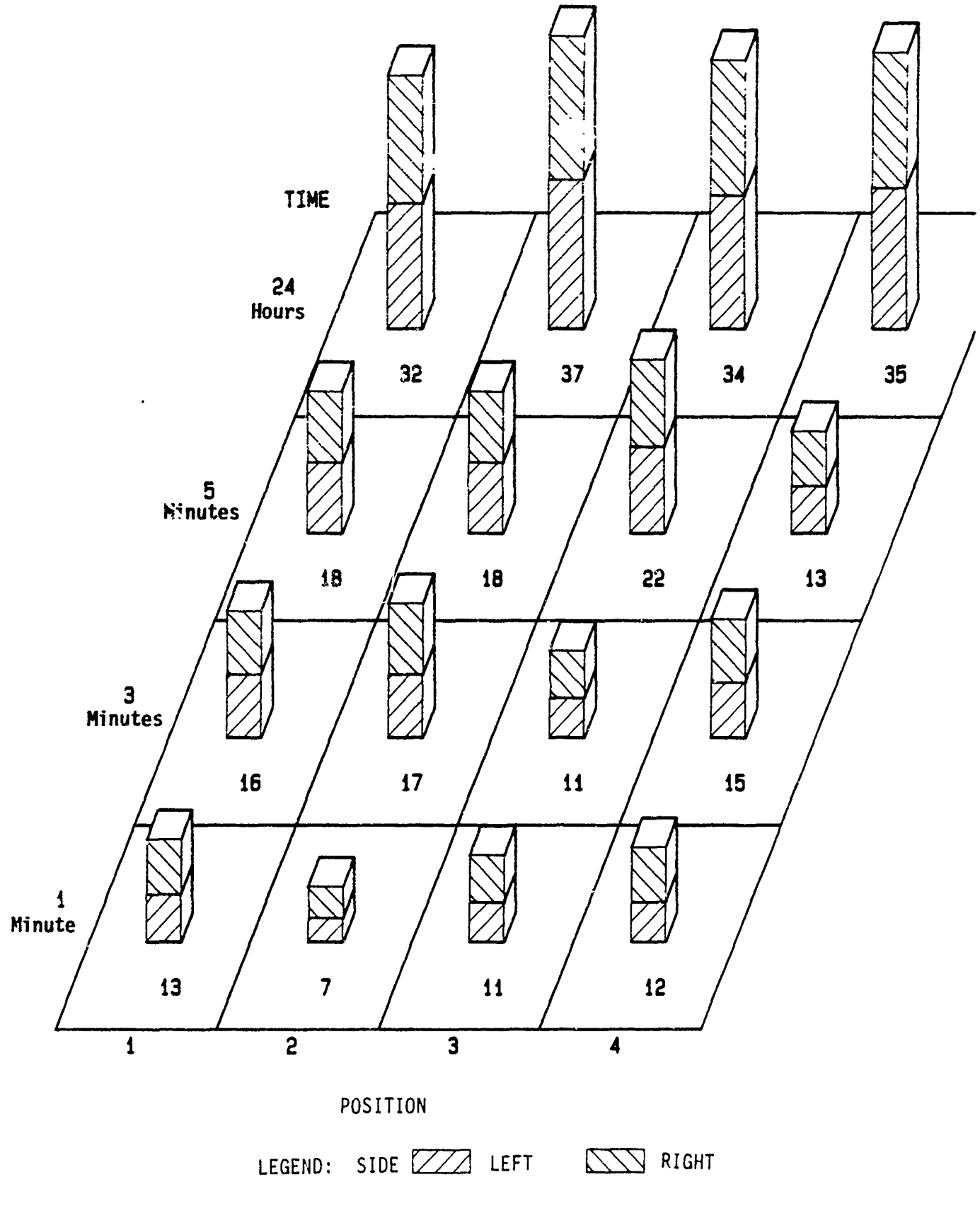


FIGURE 3.2.6. MEAN LESION AREAS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II AT 1.0, 3.0, AND 5.0 MIN, AND 24 HR AFTER DOSING

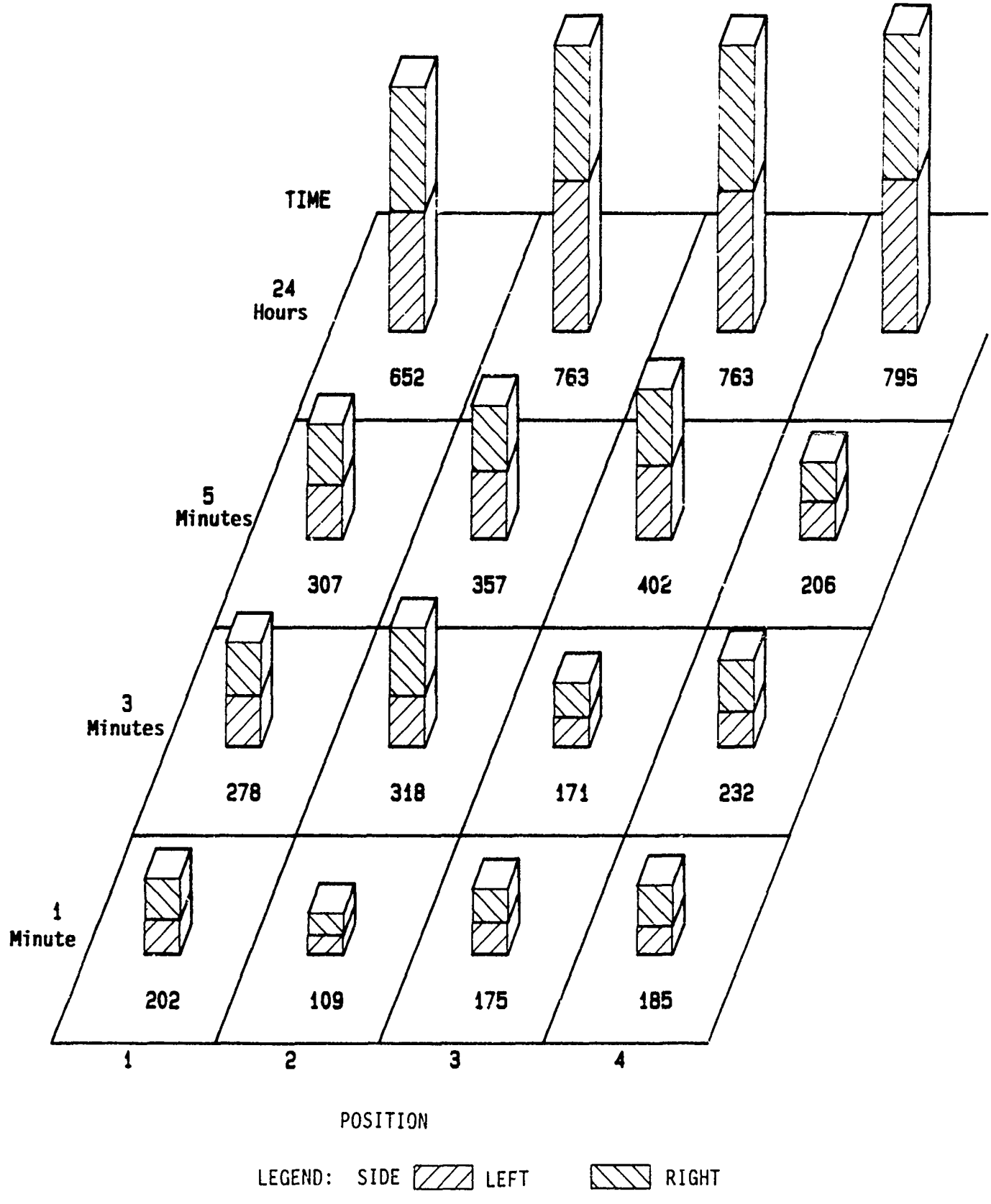


FIGURE 3.2.7. MEAN LESION LENGTHS FOR 0.5 µl of L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, and 120 SEC, AND 24 HR AFTER DOSING

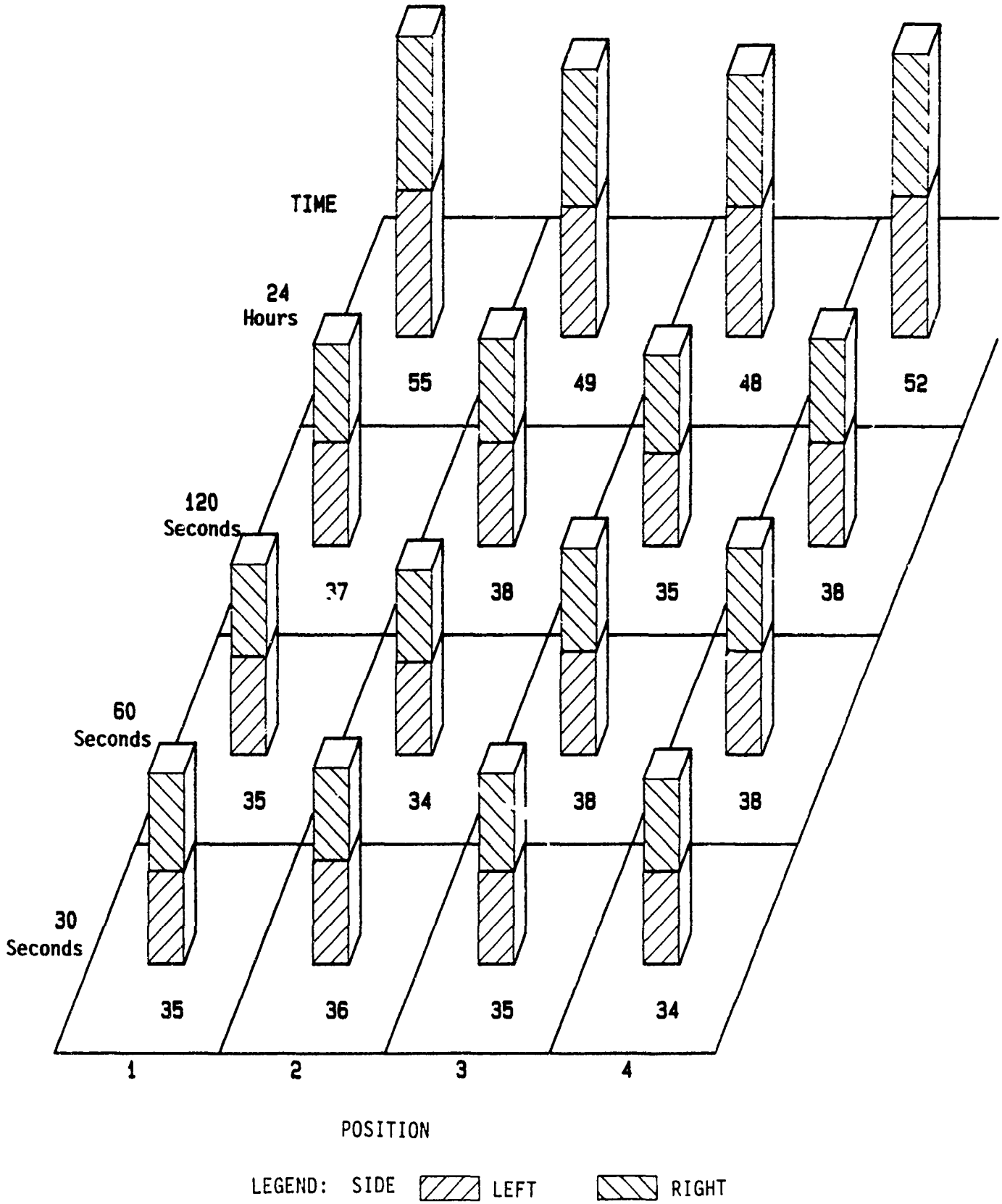


FIGURE 3.2.8. MEAN LESION WIDTHS FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC, AND 24 HR AFTER DOSING

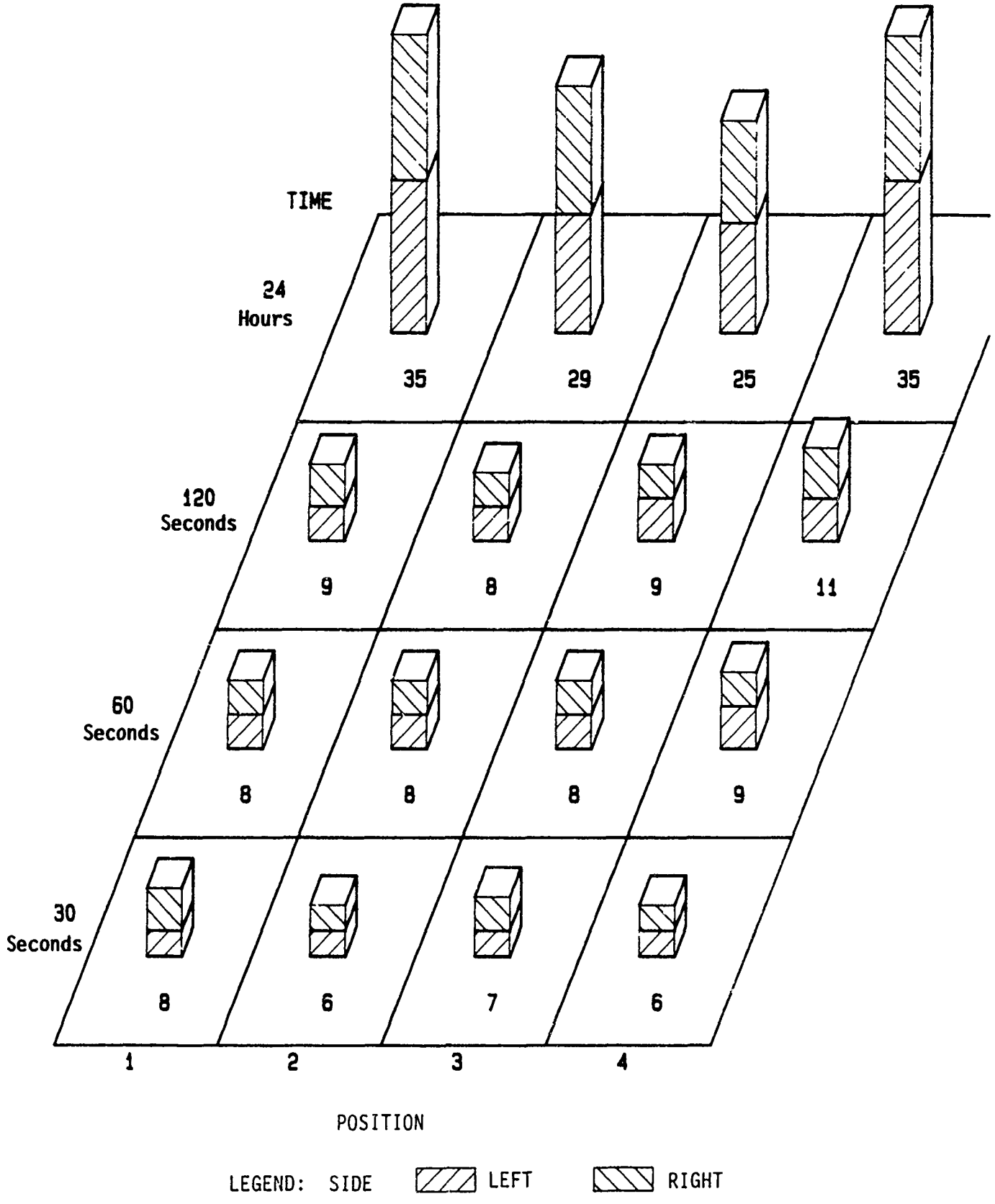


FIGURE 3.2.9. MEAN LESION AREAS FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II AT 30, 60, AND 120 SEC, AND 24 HR AFTER DOSING

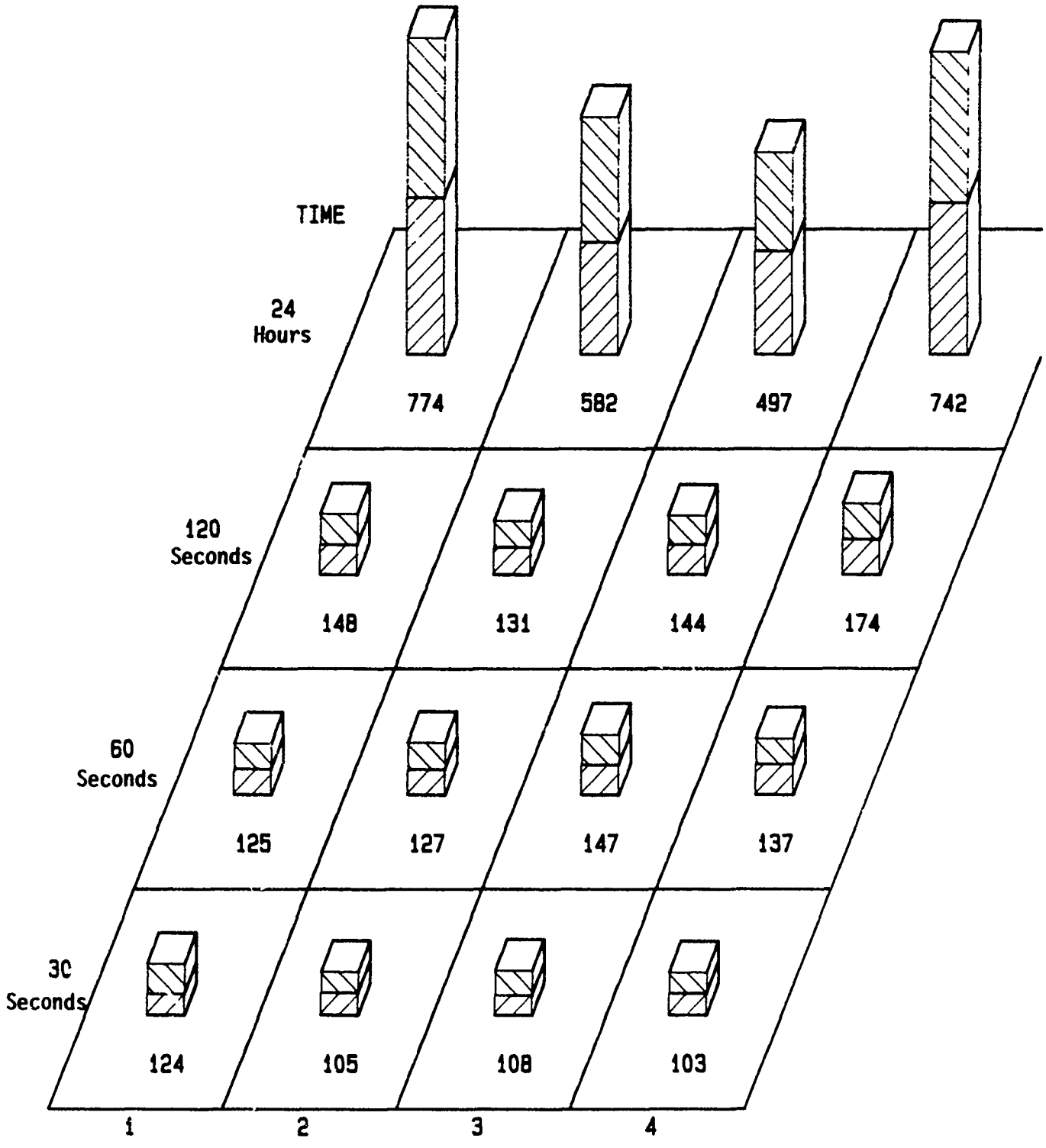


FIGURE 3.3.1. MEAN LESION LENGTHS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION

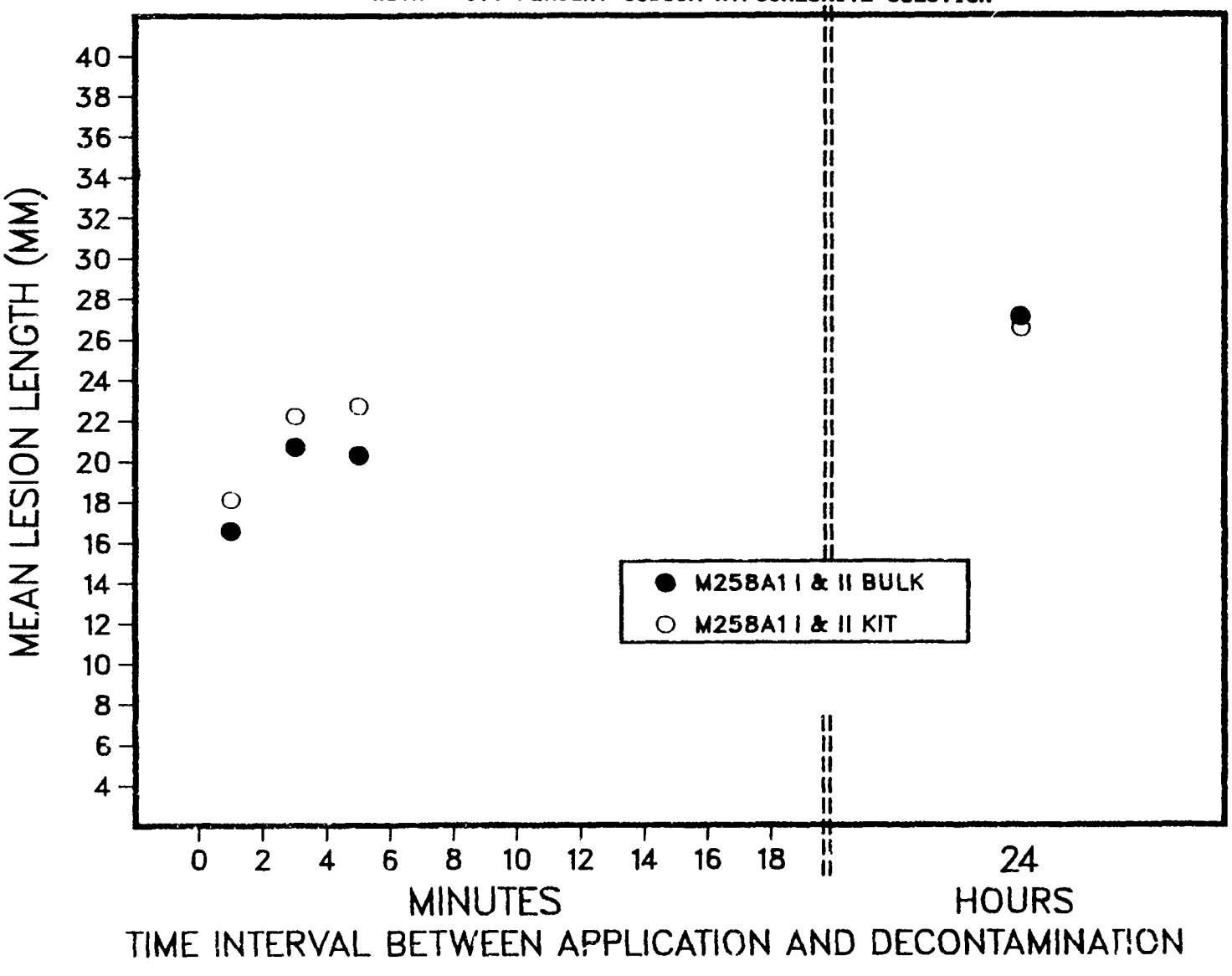


FIGURE 3.3.2. MEAN LESION WIDTHS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION

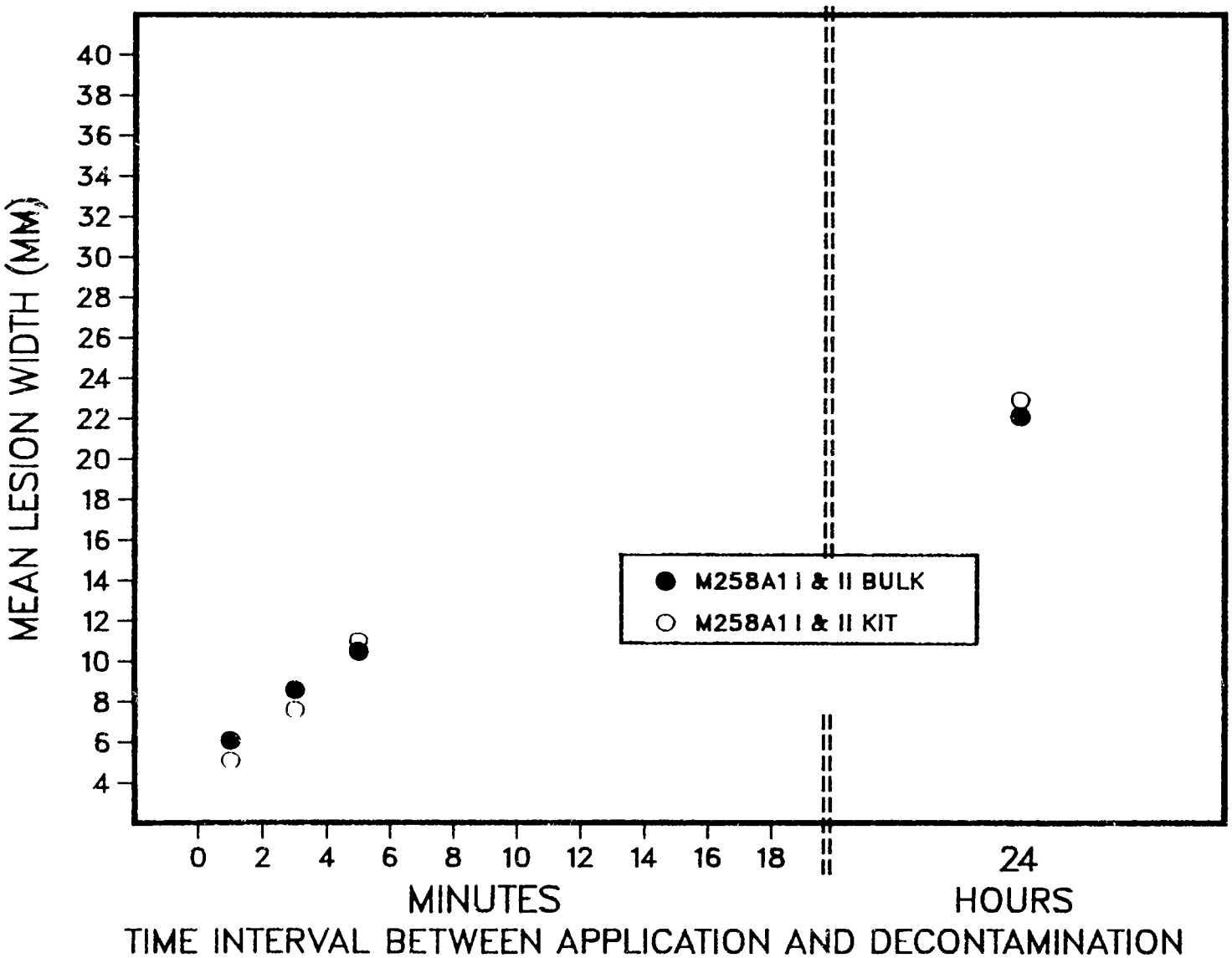


FIGURE 3.3.3. MEAN LESION AREAS FOR 0.5 µl OF HD DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 1.0, 3.0, AND 5.0 MIN FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION

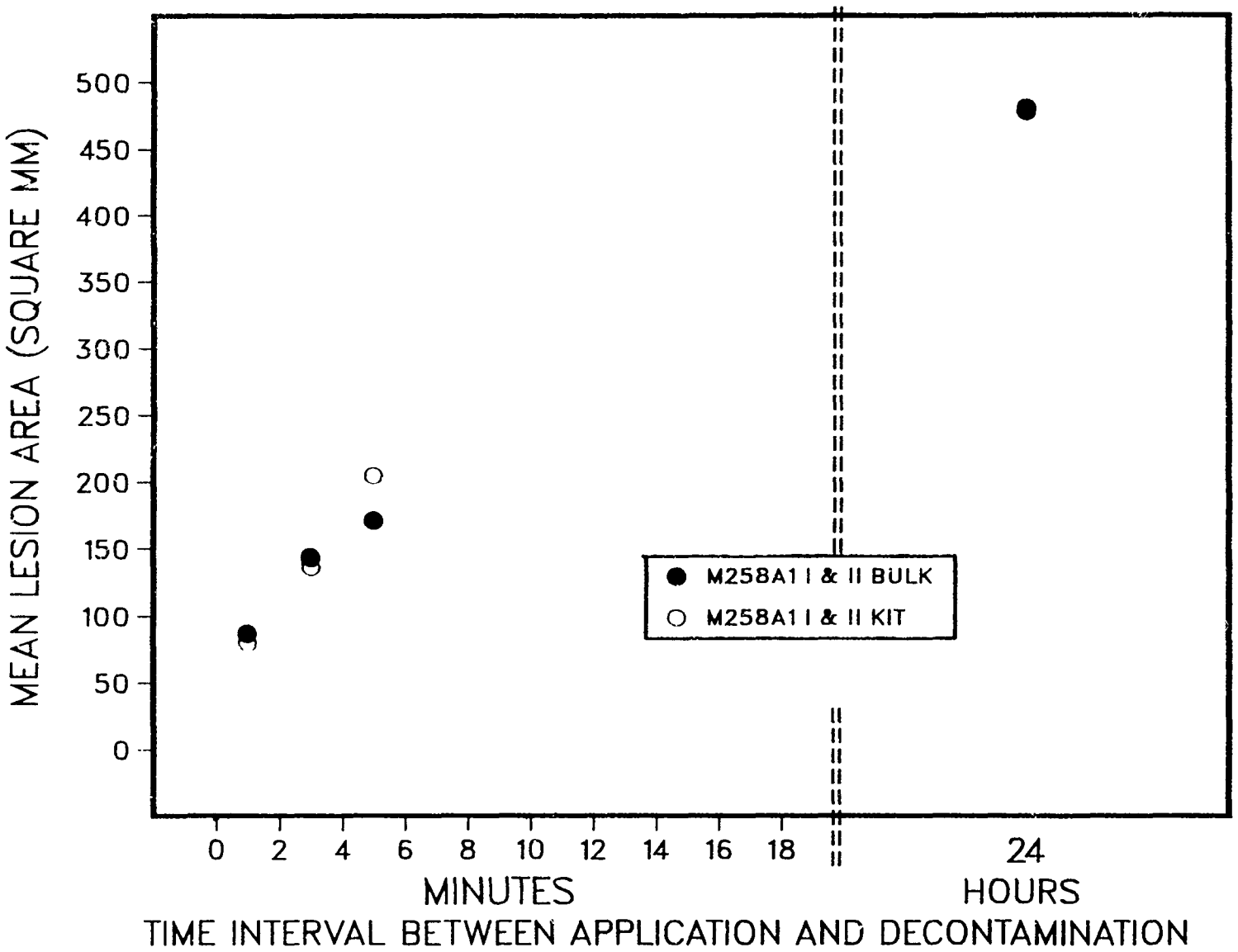
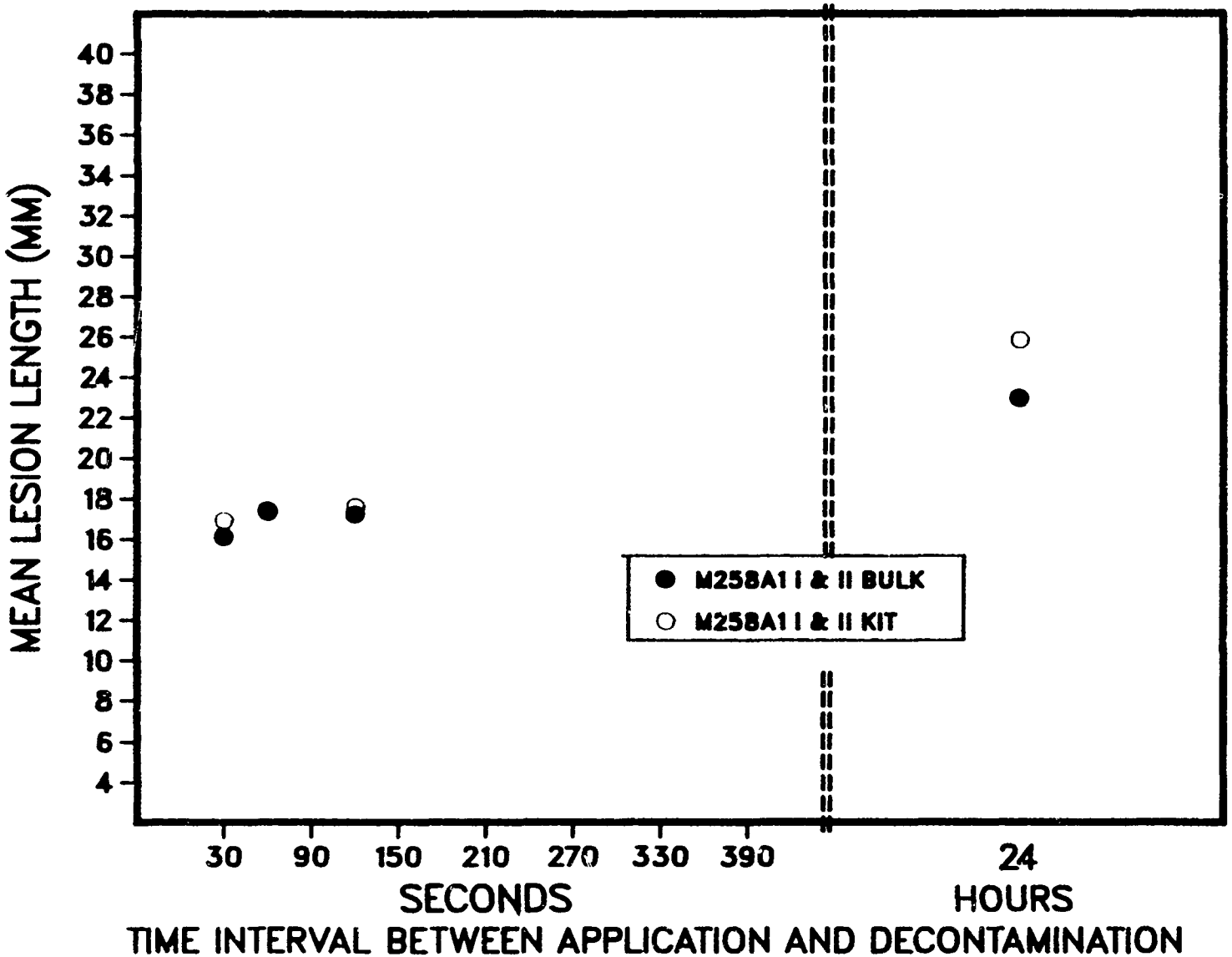


FIGURE 3.3.4. MEAN LESION LENGTHS FOR 0.5 µl of L DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION



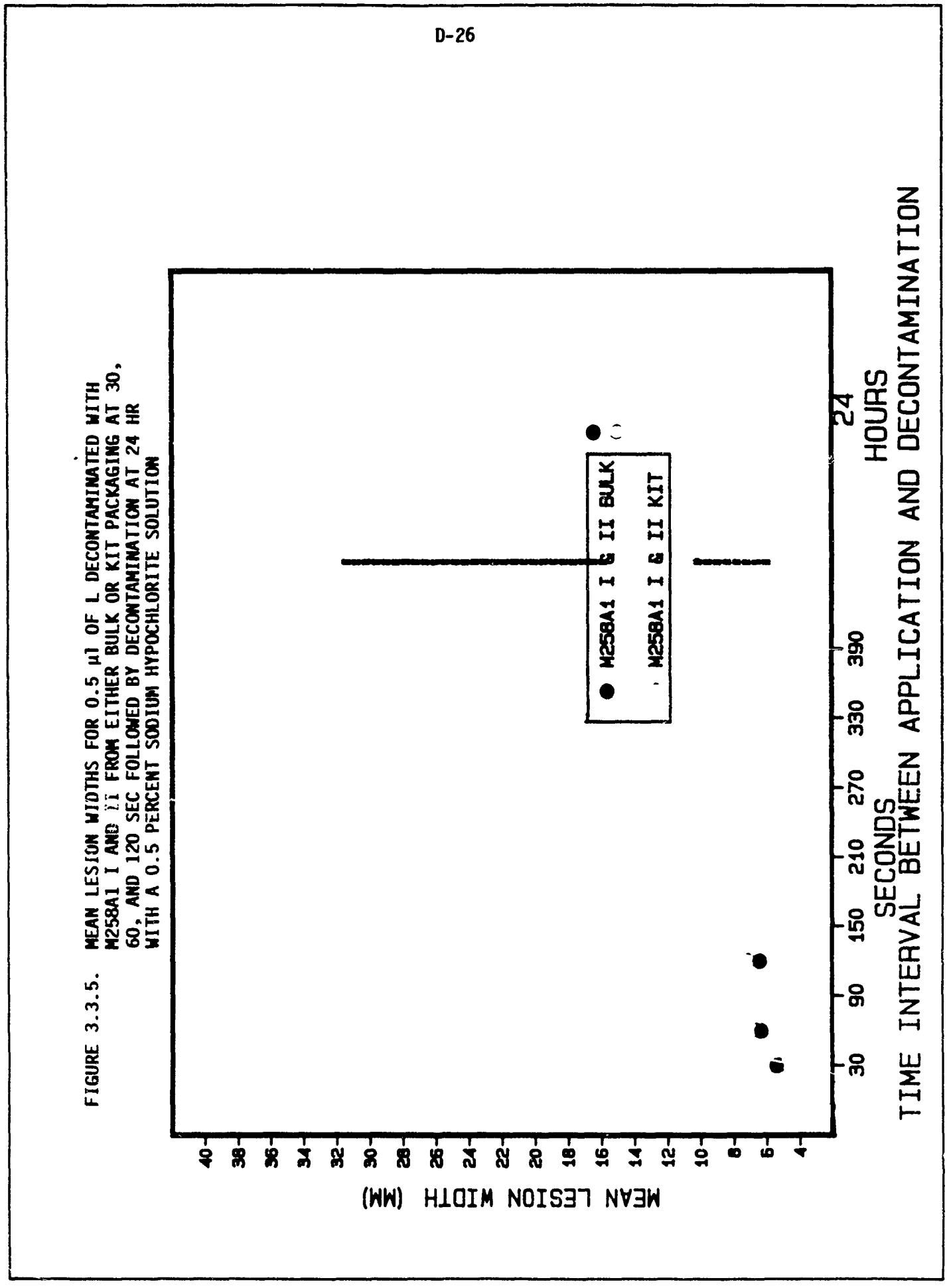


FIGURE 3.3.5. MEAN LESION WIDTHS FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION

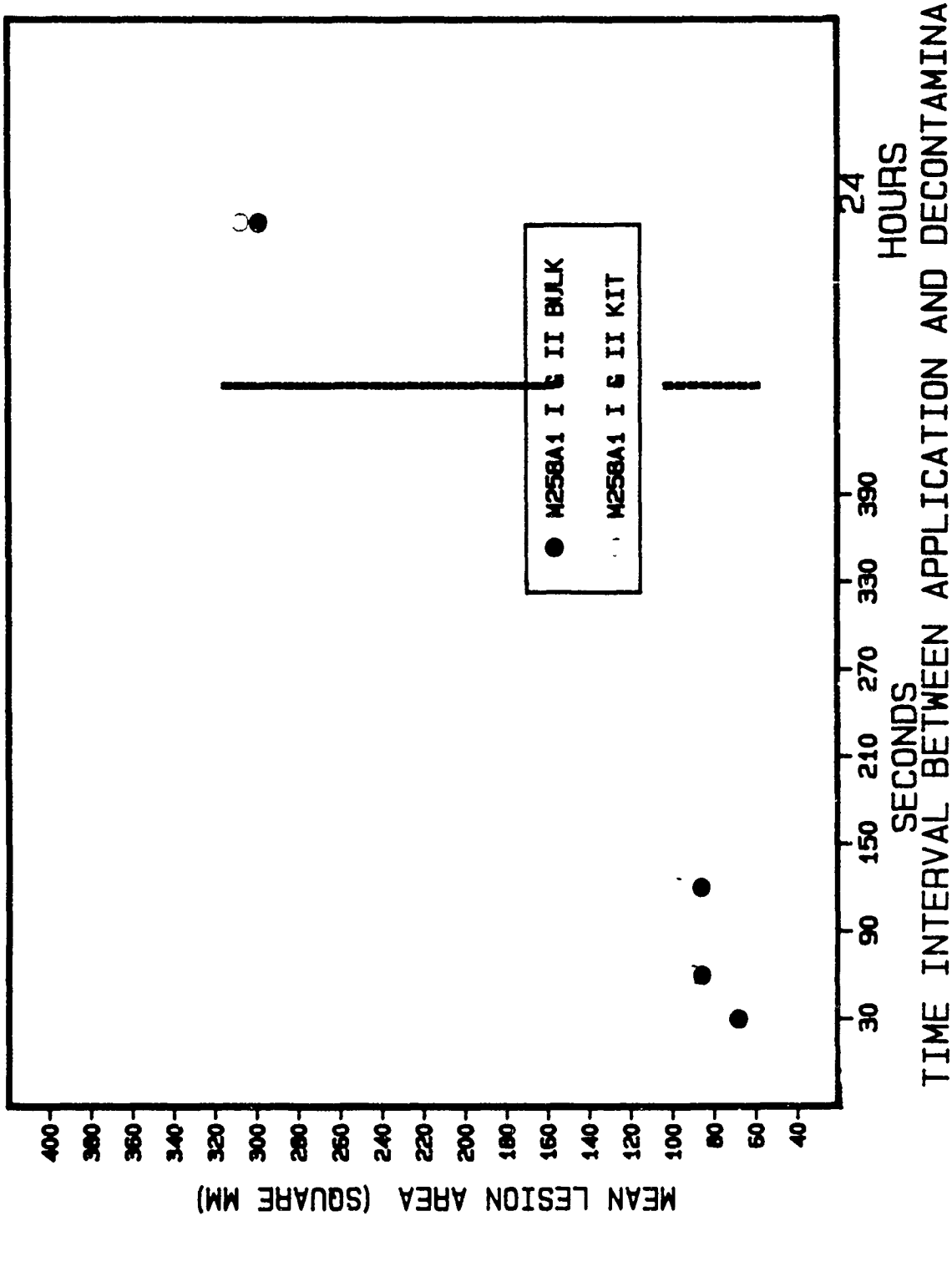


FIGURE 3.3.6. MEAN LESION AREAS FOR 0.5 µl OF L DECONTAMINATED WITH M258A1 I AND II FROM EITHER BULK OR KIT PACKAGING AT 30, 60, AND 120 SEC FOLLOWED BY DECONTAMINATION AT 24 HR WITH A 0.5 PERCENT SODIUM HYPOCHLORITE SOLUTION

FIGURE 3.4.1. OBSERVED HD LESION RATIOS (+) WITH WEIGHTED EXPONENTIAL REGRESSION CURVE (_____) AND 95% CONFIDENCE LIMITS (---)

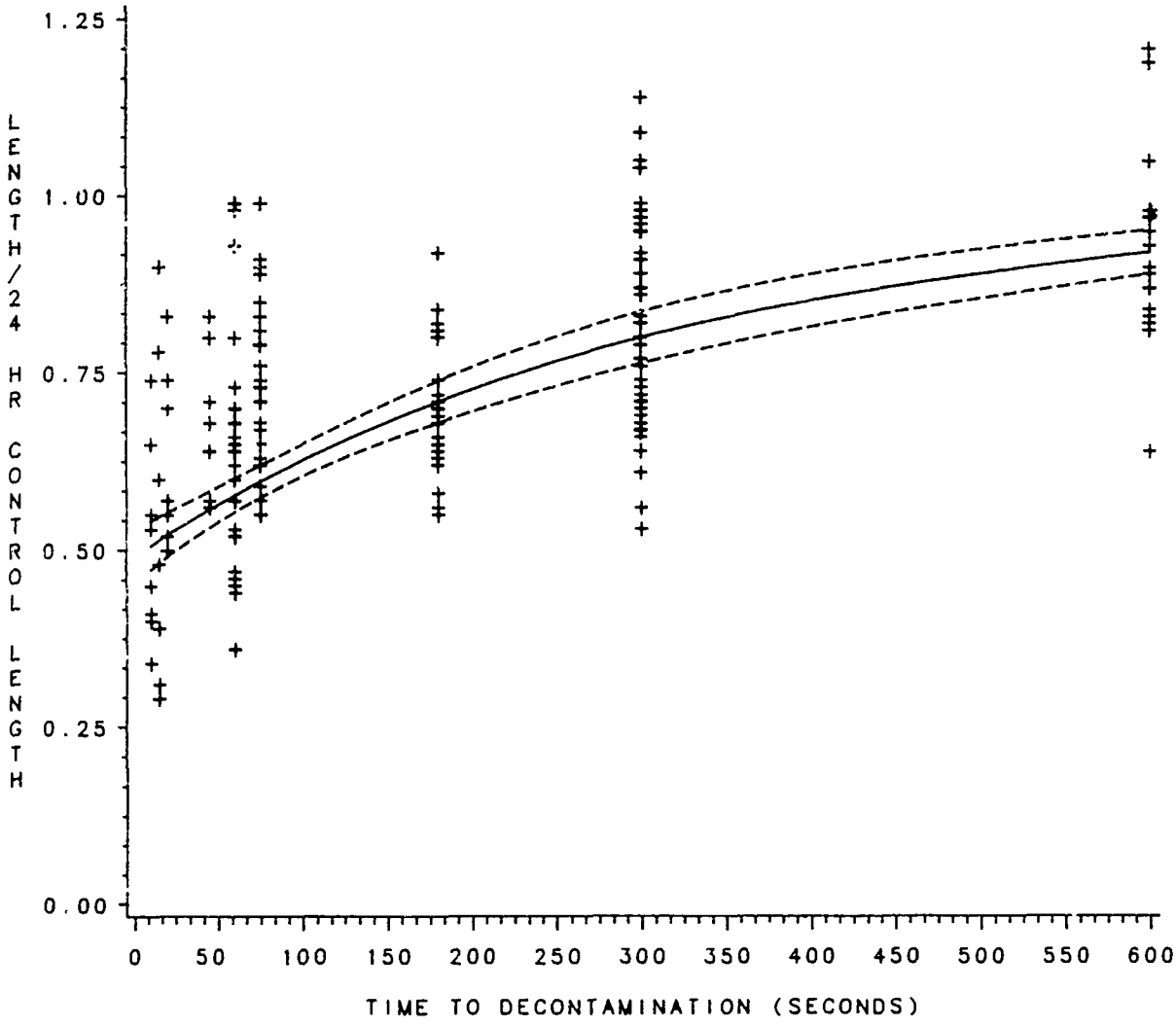


FIGURE 3.4.2. OBSERVED HD LESION GROWTH RATIOS (+) WITH WEIGHTED EXPONENTIAL REGRESSION CURVE (___) AND 95% CONFIDENCE LIMITS (---)